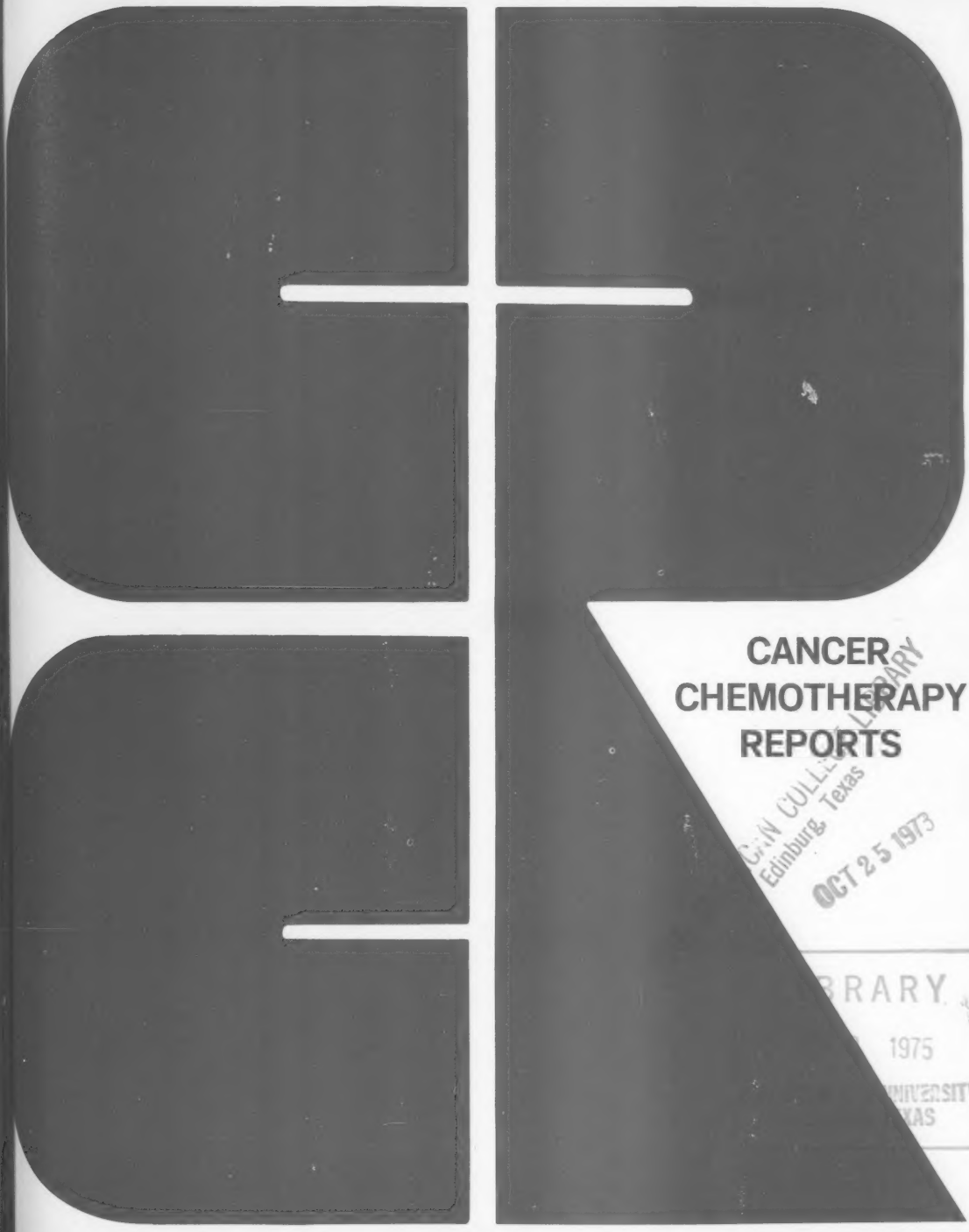

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1973.

Chemotherapy of a Spontaneous Mammary Carcinoma in Mice: Relation Between In Vitro-In Vivo Activity and Blood and Tumor Concentrations of Several Antitumor Drugs [1,2,3]

G. Balconi, A. Bossi, M. G. Donelli, S. Filippeschi, G. Franchi, L. Morasca, and S. Garattini [4]

SUMMARY

A spontaneous mammary carcinoma growing in (C3H × 020)F_1 mice was used for comparing the antitumor activity of 21 known drugs in vitro and in vivo. The tumor was cultivated in vitro in Rose chambers by utilizing the perforated cellophane-strip technique, and the activity of the drugs added at different concentrations was evaluated according to morphologic criteria (lethality and cytotoxicity). The activity of the drug in vivo was established after intraperitoneal administration at various doses and schedules of treatment. There was agreement between the in vitro and the in vivo results in only seven of the 21 drugs tested. Blood and tumor concentrations for four selected drugs (6-mercaptopurine, daunorubicin, methotrexate, and hydroxyurea) were measured after single or repeated treatments. These data were helpful in interpreting the discrepancy between the observed activity of daunorubicin in vitro and its inactivity in vivo. In fact, the peak level of daunorubicin in the blood or in the tumor hardly reached the minimal concentration found effective in vitro. It is concluded that the prediction of in vivo effectiveness by in vitro activity may be improved by a better knowledge of the kinetics and metabolism of antitumor drugs.

[Cancer Chemother Rep 57:115–124, 1973]

Various techniques have been proposed and utilized in the hope of predicting with an in vitro test the sensitivity of a given tumor to chemotherapeutic agents when administered in vivo (1–8). Thus, human tumor tissue obtained by biopsy or by surgical operation has been cultivated in vitro using several methods, including primary short-term cultures (1,2), organ cultures (5), and suspension of trypsinized tumors (4,6). Such preparations were then exposed to the action of various antitumor drugs and the most effective agent was usually selected for therapy (9,10). However, these procedures do not take into account the fact that even if a drug is very potent, it may never achieve the concentrations in vivo necessary to exert the required effect. In order to approach this problem at an experimental level, a spon-

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[2] Supported by contracts 115/1134/0-5033 from the Italian National Research Council and 079-69-1-BIA-C from EURATOM.

[3] Chemical nomenclature, NSC numbers, CAS registry numbers, and sources for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[4] Istituto di Ricerche Farmacologiche "Mario Negri," Via Eritrea 62, 20157 Milan, Italy.

taneous mammary carcinoma (SMC) developing in (C3H × 020)F_1 mice was used as a model system for human tumors (11,12). The sensitivity of this tumor to various agents was evaluated both in vitro and in vivo. Moreover, the concentrations of the selected drugs were measured in the plasma and in the tumor in the hope of finding an explanation for some of the discrepancies observed.

MATERIALS AND METHODS

Animals and Tumor

Animals.—Tumor-bearing (C3H × 020)F_1 hybrid mice were obtained from the International Center for the Provision and Study of Tumor-Bearing Animals (Prof. O. Mühlbock, Amsterdam, Netherlands). The female mice were virgin or "force-bred." The males had been castrated at an early age and treated with estrone dissolved in clear water (2 mg/liter). The mice used in the experiments were about 9 months old and weighed 25–30 g. They were kept in Makrolon cages (20 × 20 × 13 cm) at a room temperature of 22 C and a relative humidity of 60%. They had free access to water and food during the experiments.

Tumor.—Nearly all hybrid (C3H × 020) F_1 mice are known to have SMC by the age of 250 days. These tumors occur along the milk crest in both females and castrated males (11–14) and the tumors are predominantly of the adenocarcinoma type. It has been established that there is no significant difference between the tumor growth rate of the castrated male and the female mice and that the tumor is not hormone-dependent. In fact, ovariectomy and treatment with androgens do not affect the growth of the tumor at least until it has reached a palpable size (12). The growth characteristics of such tumors are reported in table 1.

TABLE 1.—Growth characteristics of spontaneous mammary carcinoma in (C3H × 020) F_1 mice

Parameters*	Value
No. of mice	
male	340
female	560
Mean diameter of single tumor (mm ± SE)	
Day 1	13.5 ± 0.5
Day 22	19.3 ± 1.7
Body weight (g ± SE)	
Day 1	30.60 ± 0.45
Day 22	33.37 ± 0.54
Tumor weight per mouse (g ± SE)	
Day 22	4.48 ± 0.81
No. of tumors per mouse (± SE)	1.87 ± 0.13

*Day number indicates time elapsed after the beginning of observation.

In Vitro Test

Establishment of cell cultures.—Under aseptic conditions the SMC was removed from the ether-anesthetized animals. The neoplastic tissue was minced into fragments of about 1 mm each and thoroughly washed with the tissue culture medium 199. Two fragments of the tissue were implanted in each Rose chamber by the perforated cellophane-strip technique (15). Culture medium 199 plus 20% calf serum was added to the chambers and incubation was started at 36.5 ±0.2 C in 95% air, 5% CO_2. Cell migration from the explants occurred between 2 and 4 days after implantation. Most of the outgrowth was formed of epithelial cells, but in a few explants only endothelial cells and fibroblasts were present. The experiments lasted no longer than 2 weeks.

Determination of the lethal concentration and the cytotoxic concentration.—One or more microscopic fields containing epithelial cells were marked with India ink on the outside of each chamber and phase-contrast pictures were taken. In each previously randomized chamber, the growth medium was replaced with fresh medium of the same composition but which contained different concentrations of the various drugs. Each experiment was performed on a tumor from a single animal and the cells' ability to reproduce was tested as described by Morasca et al (16). The same microscopic fields were rephotographed 24 hours later and the photographs were compared. The lethal concentration was the lowest concentration capable of damaging the epithelial population in a nonreversible manner as determined by the recovery of growth. The cytotoxic concentration was the lowest concentration capable of modifying the normal morphology of the

epithelial population without any definite lethal effect.

In Vivo Test

Therapeutic toxicity test.—The (C3H × 020)-F_1 hybrid mice with SMC were divided into several groups. The diameters of the tumors were measured with calipers and the tumor growth was observed for 1 week. After this time lapse those mice which did not show any change in tumor volume were excluded from the experiment. Drugs were administered on Days 1–5, 8, 10, 12, 15, 17, and 19—a total of 11 times. All of the compounds were given intraperitoneally (ip) in a volume of 0.1 ml/10 g of body weight. The drugs were dissolved in saline or in suitable solvents, or were administered as a suspension in carboxymethylcellulose (1% w/v).

If an animal had more than one tumor at the beginning of the treatment or if multiple tumors appeared in the course of the experiments, the volumes of the tumors were added. On Day 22, 15 animals were killed and the wet weight of their tumors was determined. Drug toxicity was evaluated by comparing the change in body weight from the beginning to the end of the drug administration in the treated animals with that of the control animals. The tumor-inhibition index was obtained by calculating the ratio of tumor weight in treated animals relative to controls. The inhibition of tumor growth ($P < 0.05$, "Student's t" test) without the occurrence of lethality was taken as the cutoff point for significant antitumor activity.

Biochemical Determinations

Concentrations of the following drugs were measured in the blood and in the tumor: 6-mercaptopurine, methotrexate, daunorubicin, and hydroxyurea.

6-Mercaptopurine (6-MP) was measured in the blood according to the spectrofluorometric method of Finkel (17) (recovery was 80% ±5% and sensitivity was 0.25 μg), and was measured in the tumor tissue according to a spectrophotometric method developed in this laboratory. Tumor samples were homogenized in 4 volumes of ice-cold 1.15% KCl solution. Two ml of the homogenate was transferred to a 50-ml centrifuge tube and diluted with 1 ml of distilled water and 1 ml of potassium phosphate buffer. One gram of sodium chloride was added to each tube. The extraction was performed with 40 ml of ethylacetate by shaking for 30 minutes at room temperature. After centrifugation, 35 ml of the organic phase was transferred to another 50-ml centrifuge tube that contained 3 ml of NaOH 1N. The aqueous phase was read at a wavelength of 310 mμ. The recovery was 60% ±9% and the sensitivity was 2 μg.

Methotrexate was measured in the blood according to the fluorometric method of Chakrabarti and Bernstein (18). For tumors, this method was modified as follows. Tumor samples were homogenized in 2 volumes of ice-cold 1.15% KCl solution. Seven ml of water and 2 ml of trichloroacetic acid were added to 1 ml of homogenate. After deproteinization, the determination was carried out according to the method described for blood. The recovery was always the same as for blood (90% ±4%); the sensitivity was about 0.2 μg.

Daunorubicin was measured in the blood according to the method of Finkel et al (19). For tumor tissues this method was modified as follows. After homogenization in 2 volumes of ice-cold 1.15% KCl solution, 1 ml of homogenate, 0.5 ml of water, and 0.5 ml of trichloroacetic acid were mixed and, after deproteinization, the determination was carried out according to the method described for blood. Recovery was always the same as for blood (90% ±8%); the sensitivity was about 0.1 μg for blood and 0.2 μg for tumor tissue.

Hydroxyurea was measured in the blood according to the spectrophotometric method of Bolton et al (20). For tumors this method was modified as follows. After homogenization in 2 volumes of sodium phosphate buffer (pH 7.4), 0.2 ml of trichloroacetic acid 50 (v/v) and 0.8 ml of water were added to 1 ml of homogenate. After deproteinization, 1 ml of supernatant was mixed with 0.2 ml of 4% KCl solution (v/v) and the determination was carried out according to the method described for blood. Recovery was always the same as for blood (90% ±7%) and the sensitivity was about 5 μg.

Experimental Design for Evaluating Levels and Antitumor Effects of Drugs

The evaluation of levels and antitumor effects of 6-MP, methotrexate, daunorubicin, and hydroxyurea was made using both single and multiple ip treatments in (C3H × 020)F_1 hybrid mice bearing SMC. For the single treatment the drug was administered at different dose levels: 60, 250, and 500 mg/kg of 6-MP; 2 and 100 mg/kg of methotrexate; 0.5, 10, and 25 mg/kg of daunorubicin; 750, 1000, and 2000 mg/kg of hydroxyurea. The antitumor effect was evaluated 8 days after treatment. Blood and tumor concentrations of each drug tested were determined at 5-, 10-, 20-, and 40-minute intervals after treatment to detect the peak level.

For the multiple treatment the drug was administered a total of 11 times as follows: Days 1–5, 8, 10, 12, 15, 17, and 19. Drugs were administered at the following dose levels: 60 mg/kg of 6-MP; 2 mg/kg of methotrexate; 0.5 mg/kg of daunorubicin; 750 mg/kg of hydroxyurea. On Day 22, 15 mice were killed and the wet weight of their tumors was measured; in the remaining animals blood and tumor concentrations of the drug being tested were determined on the same day (Day 22) that a 12th drug injection was given.

RESULTS

In Vitro Test

Table 2 shows the sensitivity to a group of 21 known antitumor agents of the SMC cultivated in vitro. The cytotoxic and lethal concentrations are given for each drug. It is evident that the most active compounds (cytotoxic con-

TABLE 2.—In vitro and in vivo effects of antitumor drugs on spontaneous mammary carcinoma in (C3H × 020)F_1 mice

Compounds	Lethal concentration (μg/ml)	Cytotoxic concentration (μg/ml)	T/C* tumor weight, in vivo	Correlation of in vitro and in vivo effects†
Actinomycin D	0.1	0.1	0.52	Yes
Busulfan	> 100	> 100	NS	Yes
Chlorambucil	100	100	NS	No
6-Chrysenamine	> 100	> 100	0.28	No
Cyclophosphamide	>1000	>1000	0.43	No
Daunorubicin	1	0.5	NS	No
5-Fluorouracil	> 100	100	NS	Yes
Hydrocortisone acetate	> 100	> 100	0.59	No
Hydroxyurea	>1000	>1000	NS	Yes
Mannitol mustard	100	10	NS	No
Mechlorethamine	100	100	NS	No
6-Mercaptopurine	> 500	100	0.47	Yes
Methotrexate	> 100	> 100	NS	Yes
Mitomycin C	100	10	NS	No
Sarcolysin	100	10	0.45	Yes
SPI-77	100	1	NS	No
ThioTEPA	> 100	100	NS	No
Triethylenemelamine	100	10	NS	No
Tris(1-aziridinyl)*p*-benzoquinone	0.01	0.01	NS	No
Vinblastine	0.1	0.01	NS	No
Vincristine	0.01	0.01	NS	No

*T/C = Treated/control. NS = not significant.

†Correlation based on agreement or disagreement of in vivo effect and cytotoxic activity (cutoff point, 100 μg/ml).

centration ≤ 1.0 μg/ml) are tris (1-aziridinyl)-*p*-benzoquinone, vinblastine, vincristine, actinomycin D, and daunorubicin. Another group of compounds showed moderate activity (cytotoxic concentration $\leq$ 100 μg/ml), including, in decreasing order of activity, a podophyllin derivative (SPI–77), mechlorethamine, mannitol mustard, sarcolysin, triethylenemelamine, mitomycin C, chlorambucil, 6-MP, and triethylenethiophosphoramide (thioTEPA). The remaining seven compounds showed no effect up to a concentration > 100 μg/ml and were therefore considered inactive.

In Vivo Test

Table 2 also summarizes the in vivo effects on SMC of the 21 agents. The results obtained in vivo at various doses are shown in greater detail in table 3. Among the compounds considered active in vitro, only actinomycin D was significantly effective in vivo; among the compounds considered moderately active, only sarcolysin and 6-MP showed an effect in vivo. Two compounds (cyclophosphamide and 6-chrysenamine) showed a marked effect in vivo although they were inactive in vitro. Only seven compounds of the 21 tested showed a correlation between the effect observed in vitro and the effect measured in vivo (table 2).

TABLE 3.—Antitumor treatment of spontaneous mammary carcinoma in (C3H × 020)F_1 hybrid mice

Compounds	Dose (mg/kg × 11, ip)*	No. of mice		Body weight change (%)		T/C tumor weight ratio	Deaths (%)
		Male	Female	Controls	Treated		
Alkylating agents							
Cyclophosphamide	15	5	5	+17	+17	0.58†	0
	30	8	4	+17	+ 6	0.57†	0
	60	15	8	+18	+ 7	0.43‡	0
	75	5	5	+17	+ 9	0.44‡	10
	90	8	5	+20	− 6	0.28‡	61
Chlorambucil	0.2	5	5	+11	+ 8	1.20	20
	0.4	5	5	+17	+ 9	0.66	10
	0.8	5	5	+15	+ 9	1.02	20
Mechlorethamine	0.5§	5	5	+12			90
	1.0	5	5	+11	+ 3	0.66	20
Mannitol mustard	13.5	5	5	+11	+ 9	0.70	10
	20	5	5	+12	− 8	0.65	50
	27	5	5	+12			100
Busulfan	10	6	4	+17	+14	0.87	0
	20	5	5	+15	+ 2	0.59	10
Sarcolysin	2	6	4	+23	+14	0.83	0
	4	5	5	+15	+ 2	0.66	0
	8	5	5	+15	− 9	0.45‡	0
Triethylenemelamine	0.20	8	4	+11	+ 9	0.99	0
	0.35	6	4	+23	+12	0.60†	10
	0.50	8	4	+14	− 8	0.19‡	58
ThioTEPA	2.4	5	5	+11	+10	0.91	20
Antimetabolites							
5-Fluorouracil	10	13	9	+14	+12	0.78	0
	20	13	10	+14	+14	0.82	0
	40	5	5	+15			100

TABLE 3.—Antitumor treatment of spontaneous mammary carcinoma in (C3H × 020) F_1 hybrid mice—continued

Compounds	Dose (mg/kg × 11, ip)*	No. of mice		Body weight change (%)		T/C tumor weight ratio	Deaths (%)
		Male	Female	Controls	Treated		
Methotrexate	0.2	8	4	+17	+13	0.76	0
	1.0	5	8	+17	+17	0.94	10
	2.0	5	5	+17	+10	1.10	10
6-Mercaptopurine	30	8	4	+17	+ 4	0.47†	0
	60	8	5	+17	−22	0.29‡	25
Antibiotics							
Actinomycin D	0.02	5	5	+14	+14	1.20	0
	0.05	5	5	+12	− 2	0.52†	0
	0.1	5	5	+12			65
Daunorubicin	0.5	10	10	+10	+ 7	0.73	0
	1.0	5	5	+19	+ 6	0.85	50
	2.5	5	5	+19			100
Mitomycin C	8	5	5	+14	+11	1.42	10
	12	5	5	+15			100
Alkaloids							
Vincristine	0.15	5	5	+14	+17	1.03	10
Vinblastine	0.19	10	10	+12	+13	1.05	20
Steroids							
Hydrocortisone acetate	25	5	5	+21	+12	0.67	0
	50	8	4	+13	− 4	0.59†	0
	75	8	5	+21	+19	0.73	0
Miscellaneous							
SPI-77	30	5	5	+14	+12	0.88	20
Tris(1-aziridinyl)-*p*-benzoquinone	1.0	5	5	+14	+ 9	0.88	0
	2.0	5	5	+14			100
Hydroxyurea	500	8	6	+19	+ 5	0.68	8
	750	5	5	+17	+ 8	0.47†	30
6-Chrysenamine	25	14	9	+19	+17	0.53†	0
	50	14	8	+17	+12	0.28‡	0
	100	36	22	+15	+10	0.45‡	10
	300	8	8	+13			100

*Treatment was performed by injecting the drugs on Days 1–5, 8, 10, 12, 15, 17, and 19. On Day 22, 15 animals were killed. Antitumor effect was evaluated by determining the tumor weight on that day as reported in the Materials and Methods section.

†$P < 0.05$, "Student's t" test.

‡$P < 0.01$, "Student's t" test.

§Twice daily.

Metabolic Studies

Four compounds (6-MP, daunorubicin, methotrexate, and hydroxyurea) were used to obtain information about the blood and tumor concentrations in vivo with the hope of adding another parameter for a correct interpretation of the results obtained. The results obtained for these four drugs are reported in table 4.

When a single treatment of 6-MP was given, inhibition of the tumor growth was observed only when the peak blood level was higher than 100 μg/ml—the concentration found cytotoxic in vitro. The levels in the tumor were considerably lower than the levels in the blood. Repeated treatment with a dose of 60 mg/kg (inactive as a single treatment) was effective with blood concentrations lower than 100 μg/ml, but there was 25% mortality in the group of treated animals. Blood and tumor levels were lower after repeated administration than after a single treatment.

After a single treatment of daunorubicin, even at 10 mg/kg ip, the blood level hardly reached 0.5 μg/ml (which was the level found effective in vitro) and this level was short lasting. Repeated treatments were ineffective and the blood and tumor levels did not reach 1 μg/ml (blood) or 1 μg/g (tumor).

The blood and tumor levels for methotrexate did not exceed 10 μg/ml in any instance, and the drug was inactive in vitro even at 100 μg/ml. Single treatments of hydroxyurea had no effect even at doses of 2000 mg/kg ip. With this treatment the blood and tumor levels were higher than 1 mg/ml (blood) or 1 mg/g (tumor), concentrations which were also inactive in vitro. Repeated treatments were effective, although the blood levels reached a peak of only 618 μg/ml; this effect, however, was obtained at the expense of a 30% mortality rate.

DISCUSSION

This study was designed to determine the feasibility of predicting by means of in vitro tests the efficacy of antitumor drugs on SMC in (C3H × 020)F_1 mice. The general conclusion is rather negative because the most effective drugs in vitro (vinblastine, vincristine, and tris(1-aziridinyl) *p*-benzoquinone) were inactive in vivo, while two of the drugs which were completely inactive in vitro (cyclophosphamide and 6-chrysenamine) were quite effective in vivo. It certainly does not seem desirable to rely on SMC in vitro activity for selecting the best drug for therapeutic use.

The possibility of correlating the in vitro results with the in vivo activity could be improved, however, by a better knowledge of the kinetics and the metabolism of antitumor drugs in vivo. The discrepancy between the in vitro and the in vivo effect of cyclophosphamide is only apparent because it is well known that cyclophosphamide must be transformed into active metabolites to exert its cytotoxic effect (21,22). It is possible that a similar situation also exists in the case of 6-chrysenamine. Unpublished data from these laboratories indicate that cyclophosphamide may show considerable activity in vitro on KB cells if it is incubated in the presence of liver microsomal enzymes (105.000 × g liver fractions) which are responsible for the formation of the active metabolite. These data are in good agreement with Foley's paper (23) which reported a reduction in the increase of the protein content of CCRF cell lines incubated with cyclophosphamide in the presence of liver homogenate.

The advantage in knowing the drug levels in blood and tumors is clearly indicated by the experiments carried out with 6-MP, daunorubicin, methotrexate, and hydroxyurea. In the single-treatment studies, 6-MP was effective in inhibiting tumor growth only when the peak level in the blood was higher than the concentration found effective in vitro (100 μg/ml). Daunorubicin, which is inactive in vivo, hardly reached in vivo the concentration that is active in vitro. In this respect it must be recalled that the fluorescence of daunorubicin is due to the aglycone portion of the molecule and, therefore, any metabolite containing this group could interfere with a correct biochemical determination (19). The presence of metabolites is not expected under the single-treatment conditions but it may interfere with the biochemical determination of the compound after repeated treatments (24). Furthermore, while the doses added in vitro are rather stable, as in this case where the concentrations of the drugs decreased less than 30% (unpublished data) after 24 hours, the decrease of the blood levels

TABLE 4.—Blood and tumor drug concentrations and antitumor effects of 6-mercaptopurine, daunorubicin, methotrexate, and hydroxyurea in (C3H × 020)F_1 male and female hybrid mice with spontaneous mammary carcinoma

Treatment (mg/kg, ip)	T/C tumor weight ratio	Blood concentration* after— (μg/ml ± SE)					Tumor concentration* after— (μg/g ± SE)				
		5 min	10 min	20 min	40 min	90 min	5 min	10 min	20 min	40 min	90 min
6-Mercapto-purine											
60	1.16	46.5±1.7	31.1±1.1	21.3±0.6	9.0±1.5	—	9.0±2.8	7.5±2.9	9.3±2.4	8.0±1.1	—
60 × 11	0.29‡	30.1±3.1‡	17.8±1.1‡	15.3±1.4	8.7±1.4	—	8.6±1.7	5.0±0.5†	6.6±2.9	5.0±1.0‡	—
250	0.36‡	139.6±8.2	145.4±2.8	109.8±6.3	92.1±9.0	—	36.0±4.4	58.9±1.6	56.6±1.5	34.9±0.9	—
500	toxic	344.5±4.8	330.1±4.8	—	—	—	111.4±6.9	152.9±4.1	—	—	—
Daunorubicin											
0.5	—	—	<0.2	<0.2	<0.2	—	—	<0.6	<0.6	<0.6	—
0.5 × 11	0.73	—	0.6 ±0.09‡	0.4 ±0.02‡	0.3 ±0.03	—	—	0.87±0.23	0.79±0.06	0.98±0.07	—
10	1.13	—	0.65±0.05	0.55±0.03	0.44±0	—	—	0.80±0	1.28±0.08	1.04±0.07	—
25	toxic	—	2.09±0.11	1.79±0.07	1.58±0.15	—	—	0.96±0.09	2.00±0	1.56±0.04	—
Methotrexate											
2	—	—	0.40±0.02	0.33±0.01	0.20±0.01	—	—	<0.6	<0.6	<0.6	—
2 × 11	0.76	—	0.42±0.02	0.30±0.02	0.20±0.01	—	—	<0.6	<0.6	<0.6	—
100	0.94	—	6.99±0.03	5.23±0.14	3.08±0.19	—	—	6.91±0.09	0.76±0.05	0.91±0.09	—
Hydroxyurea											
750	—	578.1 ±20.6	480.0 ±21.4	305.0 ±22.8	180.0 ±20.0	102.5 ±8.28	197.2 ±17.2	303.0 ±12.6	210.6 ±17.4	189.4 ±9.8	74.4 ±15.6
750 × 11	0.47‡	618.6 ±21.2	545.0† ±14.4	312.4 ±23.0	222.4 ±8.28	115.0 ±16.9	199.8 ±19.2	381.0† ±22.4	248.2 ±13.8	199.8 ±12.8	98.4 ±13.6
1000	0.88	843.0 ±73.2	771.8 ±92.8	770.2 ±22.6	—	—	335.8 ±56.0	399.8 ±33.0	—	359.8 ±50.6	49.8 ±8.4
2000	0.89	1658.0 ±113.4	1392.2 ±69.4	1340.4 ±19.6	—	—	730.6 ±64.0	1021.2 ±63.0	—	998.0 ±23	262.0 ±65.4

*Antitumor activity was evaluated 8 days after a single treatment. Each figure represents the average of 5 determinations from randomly chosen male and female mice. Multiple treatment was carried out by injecting the drugs on Days 1–5, 8, 10, 12, 15, 17, and 19. On Day 22, 15 animals were killed for evaluation of antitumor activity, and on the same day another injection was given to the remaining animals and blood and tumor levels were measured.

†$P<0.05$, "Student's t" test (in respect to the acute treatment).

‡$P<0.01$, "Student's t" test (in respect to the acute treatment).

in vivo was much faster for all the drugs considered. It should also be added that the drugs tested in vitro are acting on only a limited number of cancer cells compared to the number of cells in vivo.

Methotrexate and hydroxyurea reached in vivo concentrations that were inactive in vitro, which confirms the fact that the SMC utilized here is insensitive to these drugs. The results obtained in repeated treatments are more difficult to interpret.

Only daunorubicin tends to accumulate in the blood and in tumor tissue, while for the other drugs the levels obtained after 11 treatments were not different from those obtained after the first administration. For 6-MP there was actually a decrease in blood and tumor levels indicating that 6-MP may be metabolized faster with repeated treatments. 6-MP was effective in inhibiting tumor growth with repeated treatments even when the levels were about one third of those considered effective in vitro. It is known, however, that 6-MP acts only after its transformation into 6-mercaptopurineribotide (25,26), and it is possible that this metabolite accumulates in the tumor during repeated treatments.

All of these considerations suffer, however, from at least two limitations. First, it may be possible that the chosen schedule of treatment might not be optimal for all the drugs used in this study, and second, a better knowledge of the cell kinetics of this slowly growing tumor should be acquired in order to establish a more rational schedule of treatment. Drugs that are specific for certain phases of the cell cycle (eg, vinblastine and vincristine) may have been inactive in vivo for this reason. Moreover, it is also possible that the in vitro experimental conditions, such as the culture methods, the contact time, or the drug concentration, may not have been optimal for some drugs which did not reveal any in vitro activity. Although the time from implantation in vitro to treatment was short, it is also possible that the ratio between "resting" and "growing" cells would be altered in the in vitro system as compared to the in vivo situation.

Despite these limitations, it is possible that a more integrated approach which would take into account the drug metabolism and possibly the cell kinetics may permit a better understanding of the relationship between the in vitro and the in vivo activity in both experimental and clinical tumors.

LIST OF COMPOUNDS (and suppliers)

Actinomycin D: NSC-3053. Drug Research and Development (DR&D), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

Busulfan: NSC-750; CAS reg. No. 55-98-1; 1,4-butanediol, dimethanesulfonate. Burroughs Wellcome & Co., London, England.

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-. DR&D, DCT, NCI, Bethesda, Md.

6-Chrysenamine: NSC-80186; CAS reg. No. 2642-98-0. Continental Pharma, Brussels, Belgium.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate. Asta Werke AG, Brackwede, Germany.

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; daunomycin (NSC-82151). Farmitalia, Milan, Italy.

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8. DR&D, DCT, NCI, Bethesda, Md.

Hydrocortisone acetate: NSC-741E; CAS reg. No. 50-03-3; pregn-4-ene-3,20-dione, 11β,17,21-trihydroxy-, 21-acetate. Laboratori Farmaceutici Maestretti, Milan, Italy.

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1. Simes, Milan, Italy.

Mannitol mustard: NSC-9698; CAS reg. No. 551-74-6; mannitol, 1,6-bis[(2-chloroethyl)amino]-1,6-dideoxy-, dihydrochloride, D-. Medimpex, Budapest, Hungary.

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard. Simes, Milan, Italy.

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate. Burrough Wellcome & Co., London, England.

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-. DR&D, DCT, NCI, Bethesda, Md.

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7. DR&D, DCT, NCI, Bethesda, Md.

Sarcolysin: NSC-14210; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, DL-; phenylalanine mustard. DR&D, DCT, NCI, Bethesda, Md.

SPI-77: NSC-72274; CAS reg. No. 1508-45-8; podophyllic acid, 2-ethylhydrazide. Sandoz, Basel, Switzerland.

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-; triethylenethiophosphoramide. DR&D, DCT, NCI, Bethesda, Md.

Triethylenemelamine: NSC-9706; CAS reg. No. 51-18-3; *s*-triazine, 2,4,6-tris(1-aziridinyl)-. Simes, Milan, Italy.

Tris(1-aziridinyl)*p*-benzoquinone: NSC-29215; CAS reg. No. 68-76-8. Sandoz, Basel, Switzerland.

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate. Eli Lilly, Sesto Fiorentino, Italy.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1). Eli Lilly, Sesto Fiorentino, Italy.

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Pharmacologic Studies of Camptothecin (NSC-100880): Distribution, Plasma Protein Binding, and Biliary Excretion [1,2]

A. M. Guarino, J. B. Anderson, D. K. Starkweather, and C. F. Chignell [3]

SUMMARY

Distribution studies of camptothecin given to mice showed that after intraperitoneal administration, the drug rapidly cleared the plasma, kidney, and liver (half-life ≃ 30 minutes), whereas the gastrointestinal tissue levels of camptothecin were cleared very slowly (half-life ≃ 210 minutes). The determination of plasma protein binding of this drug by dialysis, in vitro displacement studies, binding to purified plasma protein fractions, and fluorescence polarization measurements showed that the agent was extensively bound and that it had a very high association constant of about 7.9×10^{-6}. Binding of camptothecin to plasma in 24 different species showed that more than 90% of the drug was bound in man, monkey, hamster, guinea pig, rat, and rabbit; 80%–90% was bound in seal and lobster; 60%–80% was bound in swine, dog, mouse, and cat; and 30%–60% was bound in goat, horse, lamb, bovine, dogfish, turtle, frog, turkey, duck, pigeon, goose, and chicken. Of the purified bovine protein fractions studied, α-globulin (fraction IV–1) demonstrated the highest degree of binding (58%). All other fractions tested bound the drug from 20% to 50%. Biliary transport studies in rats showed that this was a major route of excretion of camptothecin. The high bile/plasma ratios, the saturation properties of this transport system, and the use of anionic drugs as inhibitors of biliary transport indicated that this process occurred in part from active processes. The pharmacologic, toxicologic, and therapeutic implications of these distribution, binding, and transport studies are discussed.

[Cancer Chemother Rep 57:125–140, 1973]

Venditti (1) has reported that camptothecin (fig 1) shows a maximum therapeutic effect in the mouse L1210 leukemia system when administered by the intraperitoneal (ip) route on an intermittent schedule at the optimum dose of 40 mg/kg. The greatest increase in life-span was 105%; activity greater than 25% was taken as evidence of positive activity in this tumor test system. The best reported schedule for ip and oral treatment was q4d, Days 1, 5, and 9. The high activity of camptothecin against L1210 tumors confirmed earlier studies (2, 3) and led to the development of both fluorometric (4) and microbiologic (5) assays for the compound. However, the some-

[1] Received Dec 12, 1972; revised Apr 3, 1973.

[2] Camptothecin was supplied by Drug Research and Development, Division of Cancer Treatment (DCT), National Cancer Institute (NCI). Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

[3] Laboratory of Toxicology, Experimental Therapeutics, DCT, NCI (Dr. Guarino and Mrs. Anderson), and Laboratory of Chemical Pharmacology, Division of Intramural Research, National Heart and Lung Institute (Mrs. Starkweather and Dr. Chignell), Bethesda, Md.

what encouraging preliminary clinical findings of this drug (6) were not borne out in a phase II study against advanced gastrointestinal cancer (7).

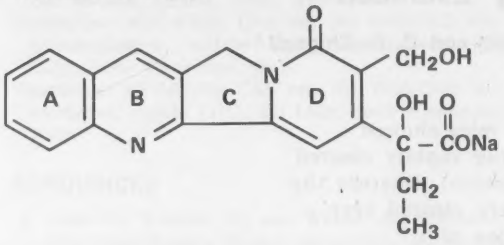

FIGURE 1.—Structural formula and ring labeling for camptothecin.

Recently, a number of investigators have described a variety of novel effects of camptothecin on macromolecular synthesis. This drug has been reported to inhibit nucleic acid biosynthesis in mammalian cells (8) and RNA synthesis in L1210 leukemia (9). From these and other reports (10–15), it would appear that the mode of action of camptothecin is significantly different from that of other inhibitors of nucleic acid synthesis which have been studied to date. Its effects on RNA metabolism are distinctly different from those of actinomycin D (14) because camptothecin selectively inhibits ribosomal and messenger RNA formation but does not inhibit the synthesis of low molecular weight RNA and mitochondrial RNA. Because of the possible clinical utility of camptothecin and the recent extensive use of this agent to study events at the molecular level, these pharmacologic studies were carried out.

MATERIALS AND METHODS

Determination of Camptothecin in Mouse Body Fluids and Tissues

The fluorometric method of Hart el al (4) was employed for the determination of camptothecin in body fluids. This method was used in tissues that had been homogenized in distilled water; the acidified 50% homogenates or body fluids were extracted with ethyl acetate. The recovery of camptothecin that had been added to the tissue prior to homogenization was greater than 90%. Some tissues, ie, intestines with contents, had to be homogenized in ice since significant losses of the drug occurred when tissue samples were heated during homogenization. The sensitivity of these assays was 0.005 μg/ml for fluids and 0.8 μg/g for tissues. In these studies, male CDF_1 mice weighing approximately 22 g were treated with 40 or 80 mg/kg of camptothecin given either ip or orally. Half-lives were determined by inspection after extrapolation to t_0.

Plasma Protein Binding

The plasma samples used in these studies were purchased from commercial sources[4] except for the samples from the following animals which we collected: monkey, seal, dog, lobster (*Homarus americanus*), and dogfish (*Squalus acanthias*). Total protein and electrophoretic patterns of plasma were conducted by standard clinical chemical methods (16). The binding studies were performed using Centriflo (Amicon Corporation, Lexington, Mass) membrane ultrafilters. In these studies sufficient camptothecin was added to 4 ml of plasma or serum to give final concentrations of 30 or 100 μg/ml. The entire assembly, consisting of the membrane ultrafilter containing the mixture, the conical support, and the empty 50-ml plastic centrifuge tube, was placed in an International refrigerated centrifuge and samples were subjected to centrifugation at 3000 rpm for 1 hour at 4 C. This procedure usually yielded about 1 ml of protein-free ultrafiltrate and this ultrafiltrate, as well as the retentate, was assayed for the drug as described above. Displacement of binding was also studied in the same manner after mixing camptothecin with either plasma or a 5% solution of purified protein, and then adding a second drug at a concentration which was 10–100-fold greater than camptothecin. Purified protein fractions were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Fluorescence Polarization to Human Plasma Albumin

Fluorescence measurements were made with

[4] Microbiological Associates, Inc., Bethesda, Md, and Rockland Farms, Gilbertsville, Pa.

an Aminco Bowman Spectrophotofluorimeter equipped with a corrected spectra attachment and an HTV–R 106 phototube. Fluorescence polarization was measured with a Glann prism assembly. The cell compartment was kept at 25 C throughout the study. Fluorescence titrations were carried out in a 10 × 10-mm quartz cell that contained a solution of 2 ml of camptothecin (1×10^{-6}M) in 0.1 M sodium phosphate buffer (pH4). A solution of human plasma albumin (2×10^{-5} M) containing 1×10^{-6} M camptothecin was added in small increments via a Hamilton microsyringe and polarization measurements were made after each addition. In order to avoid photodecomposition, the solution was exposed to the light source only during the polarization measurements.

Fluorescence polarization (p) was measured with linearly polarized radiation and was calculated from the relationship

$$p = \frac{{}^{I}vv - {}^{G}Ivh}{{}^{I}vv + {}^{G}Ivh}$$

where I is the observed fluorescence intensity, v and h refer to the orientation of the polarizer and analyzer respectively, and G is a correction factor for polarization introduced by the emission monochromator and is given by $G = I_{hv}/I_{hh}$ (17).

Biliary Transport Studies

Male Sprague-Dawley rats (240–320 g) were anesthetized with pentobarbital. Where indicated, the renal pedicles were ligated through an abdominal incision to prevent urinary excretion of drugs, and the bile duct was cannulated with a blunt 22-gauge hypodermic needle shaft attached to a 25-cm length of polyethylene tubing. After the incision had been closed, the rate of bile flow was measured for a 30-minute control period. One or two of the following drugs were administered intravenously (iv) in the femoral vein: camptothecin (0.4–400 mg/kg); probenecid (50 mg/kg); procainamide ethobromide (10 mg/kg); methotrexate (50 mg/kg); tyropanoate sodium (50 mg/kg); penicillin G (50 mg/kg); and ouabain (1 mg/kg). Each substance was dissolved in physiologic saline at a concentration such that each rat received no more than 1 ml of fluid iv. When drugs were used in an attempt to alter the rate of biliary excretion of camptothecin, they were injected into the contralateral femoral vein 5 minutes prior to administration of camptothecin. After the injections, bile was collected over 30-minute periods for a total of 90 minutes in most of the experiments. Some studies were terminated after 30 minutes. Throughout the experiments, rectal temperatures were monitored and were maintained at 37 ± 1 C by heat from an incandescent lamp suspended over the animal. At the end of the bile collection period, blood was obtained by exsanguination.

RESULTS

Mice Distribution Studies

When mice are treated ip with 40 mg/kg of camptothecin, it takes about 15 minutes (table 1) for the plasma levels to peak. After this time the plasma levels decay in a rather linear fashion with a half-life of about 27 minutes. Both the hepatic and renal tissue levels of camptothecin plateau at 15 minutes and then decay a little slower (ie, half-lives = 37–40 minutes) than the plasma levels. The intestine (with contents) levels of camptothecin, in marked contrast to the other tissues discussed, are at their maximum at 30 minutes; these levels are maintained for some time, demonstrating a very slow half-life of about 210 minutes. Since these studies lasted for only 3 hours, similar experiments were extended to 24 hours. In these longer studies, 80 mg/kg of the drug was administered in an effort to assure detectable tissue levels of the drug. Figure 2 shows that the plasma, kidney, and liver cleared the drug at about the same rate, whereas the gastrointestinal tract again demonstrated a very slow clearance of the drug with high levels noted even at 24 hours.

In order to compare drug levels after parenteral and oral routes of administration of camptothecin, the drug was administered to mice by either the ip or oral route at a dose of 40 mg/kg (table 2). This information also was desired so that tissue levels might be available to explain the reported (1) schedule dependency of camptothecin when given by the oral route to animals bearing L1210 leukemia.

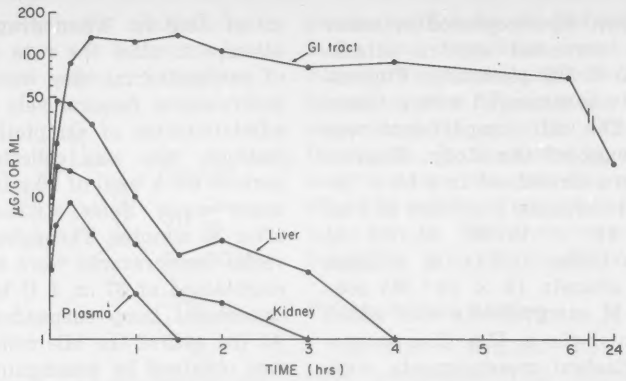

FIGURE 2.—Tissue levels of camptothecin after ip treatment with 80 mg/kg of the drug. Each point is the mean of 4 CDF_1 male mice treated after overnight fasting.

TABLE 1.—Plasma and tissue levels of camptothecin in mice*

Time (min)	Tissue levels (μg/ml or μg/g)			
	Plasma	Liver	Kidney	Intestine
1	10.40 ± 2.40	5.0 ± 1.20	0.20 ± 0.03	3.8 ± 0.40
5	21.50 ± 1.60	15.7 ± 4.37	6.60 ± 1.06	9.6 ± 2.80
15	31.20 ± 7.20	18.3 ± 3.45	6.20 ± 0.83	35.2 ± 9.60
30	19.20 ± 2.80	13.0 ± 2.24	4.90 ± 1.05	53.1 ± 4.20
60	7.00 ± 0.73	3.9 ± 0.99	1.90 ± 0.20	49.0 ± 10.20
120	1.80 ± 0.43	2.0 ± 0.33	0.70 ± 0.12	42.1 ± 4.40
180	0.65 ± 0.10	1.1 ± 0.38	0.35 ± 0.10	32.8 ± 5.40
Half-life (min)	27	37	40	210

*Male CDF_1 mice were fasted overnight and received ip injections of 40 mg/kg of the drug. All values are mean ± SD for 4 mice at each time period.

The plasma levels in table 2 show that absorption via the oral route gave plasma levels at 30 minutes which were one half those in animals treated by the peritoneal route. The levels in the plasma at this initial time were significantly different for the two modes of administration, whereas for all other times up to 24 hours the plasma levels did not significantly differ for these two groups. The intestinal levels after oral administration of camptothecin were significantly higher at 1/2, 1, and 24 hours than the levels after ip administration. As was the case in the two previous experiments, the intestinal levels of camptothecin remained virtually the same between 1/2 and 4 hours. The 24-hour intestinal levels were two fold higher after oral than after ip treatment.

Plasma Protein Binding of Camptothecin

Table 3 shows the plasma protein levels and camptothecin binding for 24 different species. In addition to typical values for total protein, albumin, and various globulin subfractions, this table contains the percent binding for camptothecin at two different concentrations, 30 and 100 μg/ml. Without attempting to segregate any particular groups of animal species, factor analyses were employed to compute the interrelations between binding, the various protein fractions, and the total protein for all species (18). In general, changing the drug

TABLE 2.—Plasma and intestinal levels of camptothecin in mice after ip or oral treatment*

Time (hrs)	Tissue levels (μg/ml or μg/g)			
	Plasma		Intestine and contents	
	ip	oral	ip	oral
½	29.0 ± 5.9	14.1 ± 5.6†	50.6 ± 4.5	86.3 ± 26.2†
1	4.4 ± 2.2	5.2 ± 3.1	56.3 ± 9.0	103.0 ± 21.5†
4	2.3 ± 1.7	0.4 ± 0.3	43.5 ± 9.6	68.6 ± 40.9
24	<0.01	<0.01	8.2 ± 1.0	18.6 ± 6.7†

*Male CDF$_1$ mice were fasted overnight and received either ip or oral injections of 40 mg/kg of the drug. All values are mean ± SD for 3–4 mice at each time period.
†Significantly different ($P < 0.05$) from ip treated group.

concentration more than threefold did not alter the percent binding. In species where the binding was altered by more than 5% by increasing the drug concentration, there tended to be lower amounts of α-globulins. For example, in the swine, goat, lamb, lobster, pigeon, goose, and chicken there were no detectable amounts of α1- and α2-globulins. In the seal, dog, cat, and duck the α1-globulin levels tended to be low, ie, between 2% and 4%. This apparent relationship between concentration-dependent binding and α1-globulin was borne out by the results of the factor analysis (bottom of table 3). It can be seen that the correlation for α1-globulin was much higher than any other interaction listed in this table. In the species of greatest interest in preclinical studies (monkey, rat, dog, and mouse), the percent of drug bound was 97.9, 95.7, 79.3, and 74.0 respectively, at 30 μg/ml of camptothecin. It is interesting to note that Schaeppi et al (19) reported that, based on a comparison of doses on a square meter basis, the acute LD50 for dogs is ten times higher than the LD50 for mice. Although certainly other factors may be operative here, it is possible that this toxicity is related to the greater amount of free drug present in the mouse.

In an effort to focus further on the protein binding, purified fractions were tested for their binding, and the results of these studies are seen in table 4. Note that as was the case for bovine whole plasma, there was only about 40% binding in the purified protein fractions (table 3). The binding to the purified fractions ranged from about 20% to 60% where the highest amount of binding was observed in the α-globulin purified fraction (IV–1). The degree of binding in α-globulin (III) and other protein fractions (III–0, IV–1, IV–7, and II) did not greatly increase when the concentration of drug was changed from 30 to 100 μg/ml. On the other hand, binding in the globulin fraction IV–7 and the albumin fraction V did show increases at the higher concentration suggesting that their binding sites had not been saturated at the lower concentration. Although purified albumin had the lowest percent binding in these studies at the 30-μg/ml level, the dominance of this protein fraction in most species suggests that the total amount of drug which is bound to albumin sites could be at least as great as is bound to the globulin sites. Hence, unlike many other drugs, camptothecin has a relative lack of specificity for any one protein fraction.

Fluorescence Polarization Measurements of Camptothecin Binding to Human Plasma Albumin

In aqueous buffer solutions (pH 7.4) camptothecin is highly fluorescent; activation spectrum of the drug showed maxima at 219, 253, 295 (shoulder), and 370 nm. The 370-nm band was chosen for this study since human plasma albumin does not fluoresce when activated at this wavelength. When a fixed amount of camptothecin (1×10^{-6} M) was titrated with human plasma albumin, the fluorescence emission maximum of the drug was unchanged, while its fluorescence intensity decreased to approximately 50% of the initial value (fig 3). The fluorescence polarization of campto-

TABLE 3.—Species differences in plasma protein levels and camptothecin binding*

Species	Total protein (g/100 ml)	Protein in fractions (%)					Binding (%)	
		Albumin	α1-globulin	α2-globulin	β-globulin	γ-globulin	30 μg/ml	100 μg/ml
Large mammals								
Human	7.4	56.5	2.4	12.1	10.6	18.4	98.3	98.7
Monkey	8.8	56.0	2.7	6.6	29.0	5.6	97.9	98.0
Seal	4.9	53.3	3.6	20.8	15.8	6.5	85.3	79.8
Swine	11.0	46.5	0.0	20.1	16.6	16.8	60.3	52.7
Goat	6.3	63.8	8.1	0.0	15.6	12.5	59.7	52.0
Horse	6.9	41.3	0.0	14.7	12.7	31.3	57.8	54.8
Lamb	7.1	50.9	0.0	18.2	7.3	23.6	44.7	33.8
Cattle	7.8	41.8	0.0	14.4	12.9	30.9	42.6	39.5
Small mammals								
Hamster	4.9	58.4	15.0	2.7	6.2	17.7	98.2	97.6
Guinea pig	3.6	59.1	24.0	4.2	2.8	9.9	93.7	86.8
Rat	6.7	47.1	27.0	0.0	18.4	7.5	95.7	95.4
Rabbit	5.7	67.9	2.2	12.4	9.6	7.9	21.6	87.3
Dog	9.0	29.2	4.1	19.9	13.3	33.5	79.3	71.0
Mouse	6.2	52.7	20.2	0.0	20.9	6.2	74.0	67.5
Cat	6.8	25.4	2.6	21.2	10.9	39.9	60.5	54.0
Fish, amphibian, and avian species								
Fish								
Lobster	2.0	79.8	0.0	17.4	0.0	2.8	81.2	55.0
Dogfish	2.8	41.8	0.0	53.5	0.0	4.7	45.6	44.3
Amphibian								
Turtle	4.1	10.5	0.0	26.4	27.2	35.9	48.0	51.0
Frog	2.7	71.2	0.0	17.3	11.5	0.0	46.0	42.0
Avian								
Turkey	4.6	46.2	0.0	12.5	14.4	26.9	53.0	55.0
Duck	3.5	51.8	3.8	5.1	15.2	24.1	52.6	41.7
Pigeon	2.3	36.8	0.0	13.1	35.8	14.3	52.3	45.5
Goose	1.7	58.6	0.0	41.4	0.0	0.0	41.0	33.3
Chicken	3.5	57.1	0.0	15.6	27.3	0.0	29.4	7.5
Correlation	0.296	0.273	0.554	−0.477	−0.109	−0.134	1.0	—

*Protein and binding assays were conducted as described in the Methods section at both 30- and 100-μg/ml concentrations of camptothecin. Protein assays are typical results from individual species as collected or purchased. Binding results are mean of 4 experiments at each concentration.

thecin, which was 0.013 in the absence of plasma albumin, increased to a value of 0.240 when the drug was completely bound to the protein (fig 3). When the concentration of human plasma albumin was increased twenty-fold, the titration curve became almost linear and showed a distinct break at a protein/drug ratio of 1.1 (fig 3). This indicates that there is only one camptothecin binding site per human plasma albumin molecule. The binding of camptothecin to human plasma albumin can be expressed in terms of the saturation fraction ($\bar{s}$), which represents the number of moles of ligand bound per mole of total ligand. The

TABLE 4.—Binding of camptothecin to purified protein fractions (bovine)*

	Binding (%)	
	30 μg/ml	100 μg/ml
α-Globulin, fraction IV–1	58.0 ± 1.17	56.7 ± 1.30
β-Lipoprotein, fraction III–0	47.3 ± 0.65	45.0 ± 1.00
β-Lipoprotein, fraction IV–1	36.0 ± 1.91	6.8 ± 1.92
Globulin, fraction IV–7	33.3 ± 0.00	40.0 ± 0.00
α-Globulin, fraction III	33.3 ± 0.00	3.8 ± 2.80
γ-Globulin, fraction II	33.3 ± 0.95	35.5 ± 2.18
β-Lipoprotein, fraction IV–	26.2 ± 4.34	22.5 ± 0.00
Albumin, fraction V	23.6 ± 1.46	27.0 ± 3.00

*Mean value ± SD of 4 experiments each. Purified proteins were made up as 5% solutions in 0.85% saline with gentle agitation and were run at two different concentrations (30 and 100 μg/ml) of camptothecin.

value of $\bar{s}$ may be calculated from the data in figure 3 by means of the equation

$$\bar{s} = 1 - \frac{I}{Io}\left(\frac{P_{max} - P}{P_{max} - P_{min}}\right) \qquad 0 \leq S \leq 1 \qquad (1)$$

where p = polarization measured at a given point in the titration, P_{min} = polarization of camptothecin in the absence of human plasma albumin, P_{max} = polarization of camptothecin in the presence of a large excess of plasma albumin, Io = fluorescence intensity of camptothecin in the absence of human plasma albumin, and I = fluorescence intensity of the camptothecin-human plasma albumin solution (20). Since there is only one ligand binding site per albumin molecule, the association constant K for the interaction can be obtained from the relationship

$$\bar{s}/P = K(1 - \bar{s}) \qquad (2)$$

where P is the concentration of unbound protein. The value of P can be calculated from the equation

$$P = P_o - \bar{s} A_o \qquad (3)$$

where P_o = total concentration of protein and A_o = total concentration of camptothecin. Figure 4 shows the data of figure 3 plotted according to equation 2, with $\bar{s}$ calculated by means of equation 1. The association constant for the interaction can be calculated from the slope of the line in figure 4 to be 7.9×10^6 M^{-1}.

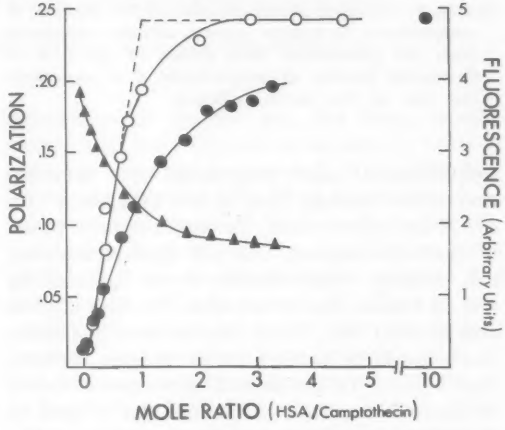

FIGURE 3.—Effect of human plasma albumin on the fluorescence intensity (▲———▲) and polarization (●———●) of camptothecin (1×10^{-6} M) and on the polarization (○———○) of camptothecin (2×10^{-5} M). All solutions contained 0.1 M sodium phosphate buffer (pH 7.4).

Fluorescence polarization provides a rapid and convenient method for monitoring drug-drug interactions that may occur at plasma albumin binding sites. For example, when phenylbutazone was added to a mixture of human plasma albumin and camptothecin, the fluorescence polarization of the latter remained unchanged (fig 5). On the other hand, the addition of dicoumarol caused a progressive decrease in the fluorescence polarization of

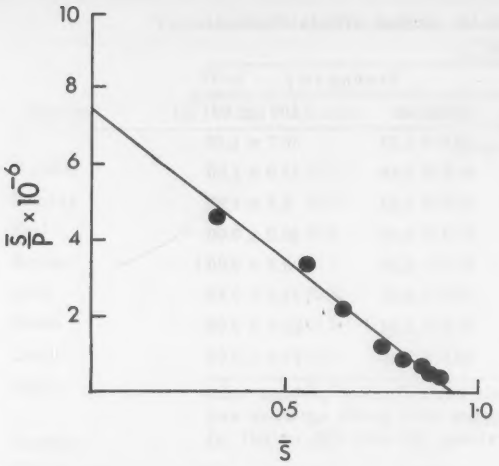

FIGURE 4.—Modified Scatchard plot of the binding of camptothecin to human plasma albumin calculated from the polarization data shown in fig 1. $\bar{s}$ = Saturation fraction of camptothecin, P = concentration (M) of free plasma albumin.

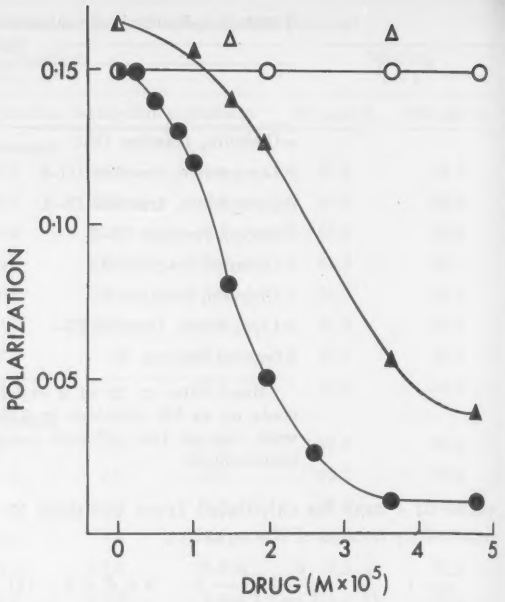

FIGURE 5.—Effect of phenylbutazone and dicoumarol on the fluorescence polarization of camptothecin bound to either human plasma or isolated human plasma albumin. Solutions contained camptothecin (1×10^{-5} M) and either human plasma albumin (1×10^{-5} M) or a sample of human plasma diluted so that it contained 1×10^{-5} M albumin. The concentration of added phenylbutazone or dicoumarol is given along the abscissa. The circles show the effect of phenylbutazone (○——○) and dicoumarol (●——●) on the polarization of camptothecin bound to isolated plasma albumin. The triangles show the effect of phenylbutazone (△——△) and dicoumarol (▲——▲) on the polarization of camptothecin bound to a sample of plasma.

camptothecin until eventually the polarization value reached that of the free drug (fig 5). These experiments demonstrate quite clearly that dicoumarol, but not phenylbutazone, can displace camptothecin from its binding site on human plasma albumin. Further studies also showed that these displacement measurements could be carried out on plasma samples (*see* below). In the second experiment shown in figure 5, a sample of plasma was diluted so that the concentration of albumin was the same as in the first experiment. The initial polarization value was somewhat higher, suggesting that plasma proteins other than albumin may also bind camptothecin. This is in agreement with the above studies (table 4) where it is noted that camptothecin also binds to the globulin fractions. Once again it was found that phenylbutazone could not displace camptothecin from its protein binding sites (fig 5). Although dicoumarol did displace camptothecin, it was less effective in the diluted plasma than in the isolated albumin system (fig 5). This observation again suggests that the camptothecin was bound to other plasma proteins in addition to albumin.

Binding of Camptothecin to Human Plasma in the Presence of Various Drugs

The percent of bound camptothecin was determined in human plasma using the Amicon ultrafiltration system discussed previously. The drugs and concentrations employed are indicated in table 5 where the concentration of camptothecin was 30 μg/ml. Since camptothecin is an acid, most of the drugs tested for their ability to inhibit the binding of camptothecin were anionic drugs. It can be seen that while the low variability inherent to this method allowed for the demonstration of signifi-

TABLE 5.—Effect of various components on the in vitro binding of camptothecin to human plasma*

Compound added	Percent camptothecin bound at indicated concentration of compound added	
	300 μg/ml	3000 μg/ml
Probenecid	99.30 ± 0.14	97.71 ± 0.07†
Procainamide ethobromide	—	97.73 ± 0.30†
Methotrexate	98.72 ± 0.10	94.20 ± 0.52†
Tyropanoate sodium	98.78 ± 0.31	97.38 ± 0.07†
Penicillin G	—	96.98 ± 0.56†
Ouabain	—	97.80 ± 0.01†
Dicoumarol	98.69 ± 0.14	73.98 ± 0.82†
Phenylbutazone	—	98.75 ± 0.09

*Camptothecin was added to human plasma at a concentration of 30 μg/ml; binding was 98.29% ± 0.44% (mean ± SD for 8 ultrafiltration experiments done in absence of any other drugs). The various drugs were added at a 10-100-fold greater concentration than camptothecin (300 and 3000 μg/ml) and results are mean ± SD for 4 experiments at each level.

†Indicates significantly decreased ($P < 0.05$) binding compared with camptothecin alone.

cantly decreased binding of camptothecin for all drugs at the higher concentration tested, the greatest differences occurred for dicoumarol and methotrexate. As had been demonstrated by the fluorescence polarization studies, dicoumarol was more effective in displacing camptothecin from its binding sites. It is important to note that drugs which are highly bound themselves, ie, tyropanoate sodium, probenecid, penicillin G, and phenylbutazone, did not alter the binding of camptothecin very much even when they were present in concentrations 100-fold greater than camptothecin. One basic compound, procainamide ethobromide, and one neutral compound, ouabain, were also tested at concentrations 100-fold greater than camptothecin and neither of these were found to efficiently alter the binding of camptothecin.

Biliary Transport of Camptothecin in the Rat

In order to establish the identity of the material being measured by the fluorescence assay (4), two different thin-layer chromatographic systems were employed (table 6) in order to verify the migration properties of the material appearing in the bile. In these two systems only one spot appeared and this spot had an R_f value identical to that of the pure drug. When 40 mg/kg of camptothecin is administered iv to the rat, the drug rapidly appears in the bile, and as is seen in table 7, most of the drug is excreted during the first 30-minute interval. The drug continues to be excreted and occurs at high levels in the bile even after 90 minutes. The liver/plasma ratio at 90 minutes is almost unity whereas the bile/plasma ratio is almost 200. This is suggestive of active transport into the biliary system. In this same time interval, 80% of the administered drug appears in the bile of animals that have been renal ligated. Since these animals have been surgically manipulated to exclude the possibility of urinary excretion, a comparison was made to study the relative excretion rates in the urinary system versus the biliary system by comparing renal-ligated rats with intact rats. In table 8, it can be seen that for the time interval under study there were no significant differences between the two groups of animals. There were some obvious trends in the intact animals suggesting that the kidneys are involved in the excretion of camptothecin, since about 10% of the administered dose appeared in this compartment. However, we were unable to demonstrate a statistically significant difference between these two groups. In the renal-ligated animals the bile/plasma ratios are considerably

higher than in the intact animals also suggesting some role, although apparently minor, for the renal compared with the biliary excretion of camptothecin. It is further noted from the urine/plasma ratio that there is a concentration gradient here but that it is much less extensive than occurred for the bile/plasma ratio in this same group of animals. It would therefore appear from considering these ratios that the biliary compartment dominates about fourfold over the urinary compartment even in intact animals.

In order to assess the saturability of the biliary transport system, the concentration of camptothecin in bile was measured over a 1000-fold range of administered doses. Figure 6, which is a double-logarithmic plot (21), shows that while the plasma concentration varies continuously over this range, the liver concentration follows the range very closely. The intestinal values are one log unit, ie, 10%, those of plasma, suggesting that the concentration of drug in this tissue occurs at a level approximating that of the extracellular space. It is particularly interesting to note that, except for the higher plasma concentration of about 100 μg/ml, there is a direct relationship between the plasma and biliary levels. The biliary levels are about 1000 times those of

TABLE 6.—Thin-layer chromatographic identification of camptothecin in rat bile*

	R_f values	
	System I	System II
Standard camptothecin	0.69	0.49
Standard camptothecin + control bile	0.68	0.50
Bile from camptothecin-treated rat	0.70	0.50

*Plates: silica gel F-254 (Brinkman Distributing Co.). System I = chloroform/ethanol (15:1); system II = ethyl acetate/isopropanol (1:1). Rats were treated iv with 40 mg/kg of camptothecin, and bile was collected as described in the Methods section.

TABLE 7.—Biliary excretion of camptothecin in renal-ligated rats*

Time period after injection (min)	Camptothecin	
	μg/ml or μg/g	μg/30 min
Bile		
0–30	9610 ± 749	5981 ± 690
30–60	2270 ± 616	1106 ± 172
60–90	848 ± 371	400 ± 136
90 minutes		
Plasma	5.1 ± 2.4	
Liver	5.5 ± 4.4	
Ratio		
Liver/plasma	1.1	
Bile/plasma	196	
Dose excreted (%)	80.2	

*Rats were treated iv with 40 mg/kg of camptothecin. Values are mean ± SD for 4 animals.

TABLE 8.—Comparison of 30-minute biliary excretion of camptothecin in intact and renal-ligated rats*

	Camptothecin value	
	Renal-ligated rats	Intact rats
Bile, μg/ml	14,990 ± 1667	13,638 ± 2080
Bile, μg/30 min	12,749 ± 2562	11,132 ± 1961
Plasma, μg/ml	49.2 ± 14.8	57.7 ± 34.3
Urine, μg/ml	—	3785 ± 2462
Bile/plasma ratio	325	290
Urine/plasma ratio	—	67
Dose Excreted (%)		
In bile	56.6 ± 11.8	46.4 ± 4.6
In urine	—	10.7 ± 6.7

*Rats were treated iv with 80 mg/kg of camptothecin. Values are mean ± SD for 4–5 rats/group. No statistically significant differences ($P < 0.05$) were observed for any of these parameters.

the plasma, suggesting active transport which is not saturable until the point in figure 6 where some flattening of the line does occur. In figure 7 it is noted that there is a linear relationship between the dose of camptothecin and the rate at which the drug appears in the bile over the 30-minute collection period. Since there is a direct proportion between the administered dose and the quantity excreted over 30 minutes but there is some deviation from this linearity in terms of concentration (fig 6), some processes other than active transport and diffusion may be operating. One possibility is that large doses of camptothecin are choleretic. In order to investigate this possibility, the choleretic response was calculated for this 1000-fold range of doses and these results appear in table 9. In this table a positive choleretic response is indicated by figures significantly greater than 100, as occurred for the four higher doses. In particular, any dose greater than 40 mg/kg caused a considerable increase in the actual amount of bile secreted in the 30-minute interval under study. Even this process showed some evidence of saturability, since comparison of the 40- and 400-mg/kg doses indicates only a threefold increase in the amount of bile secreted in 30 minutes. Nonetheless, these factors taken together suggest that the plateau seen in figure 6 is the result of the dilution effects of having a greater volume of bile in the presence of camptothecin which has not been secreted into the bile at a proportionally greater concentration.

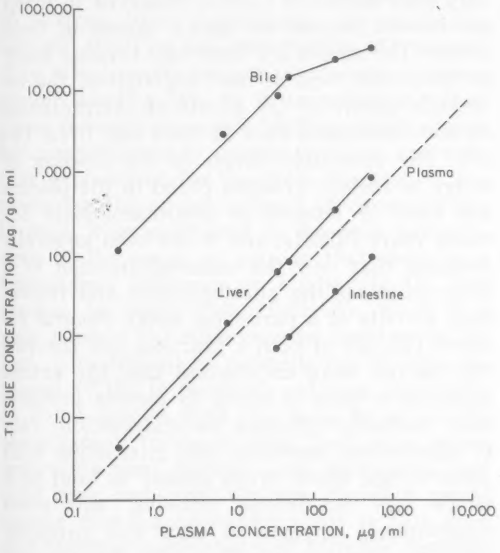

FIGURE 6.—Double-logarithmic plot of tissue levels of camptothecin vs plasma levels. Rats were treated iv as described in the Methods section.

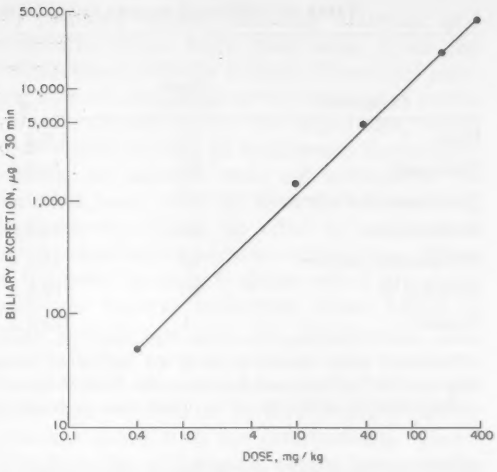

FIGURE 7.—Amount of camptothecin in bile vs administered dose. Rats were treated iv as described in the Methods section.

In the next series of experiments, especially for the drugs which apparently had an effect on displacing camptothecin in the in vitro binding experiments, agents were tested for

TABLE 9.—Choleretic response of the rat to camptothecin

Dose (mg/kg)	Choleretic response*
0.4	96.0 ± 9.8
10.0	106.3 ± 7.0
40.0	132.0 ± 14.5†
80.0	153.6 ± 5.9†
200.0	189.3 ± 33.2
400.0	198.3 ± 33.9

*Calculated as $\frac{\text{0–30-min bile vol}}{\text{30-min control bile vol}} \times 100 \pm \text{SD}$.

Three or 4 animals were used at each dose and were treated iv as described in the Methods section.

†Significantly different ($P < 0.05$) from choleretic response of previous dose.

TABLE 10.—Effect of various drugs on the biliary excretion of camptothecin in the rat*

Compound	Dose (mg/kg)	30-Min biliary excretion values of camptothecin		
		μg/ml	μg/30 min	Dose excreted (%)
None	—	102.0 ± 9.0	55.0 ± 8.0	56.1 ± 6.0
Probenecid	50	34.0 ± 6.0†	21.0 ± 7.0†	25.0 ± 6.9†
Procainamide ethobromide	10	116.0 ± 16.0	58.0 ± 15.0	55.2 ± 13.2
Methotrexate	50	51.0 ± 3.7†	39.5 ± 7.1†	33.8 ± 5.4†
Tyropanoate sodium	50	49.3 ± 17.6†	33.4 ± 18.3	29.2 ± 15.8†
Penicillin G	50	69.6 ± 11.1†	54.1 ± 4.2	48.4 ± 6.4
Ouabain	1	125.0 ± 9.2†	61.7 ± 11.2	49.5 ± 9.4

*Animals were treated iv with 0.4 mg/kg of camptothecin 5 minutes after administration of the indicated drug via the contralateral femoral vein. Each value is mean ± SD for 4 rats/group.
†Significantly different ($P < 0.05$) from animals treated with camptothecin alone.

effects on the biliary excretion of camptothecin. Rats were treated with 0.4 mg/kg of camptothecin after treatment with the agents indicated in table 10. Probenecid, methotrexate, tyropanoate sodium, and penicillin G significantly decreased the concentration of camptothecin in the 30-minute bile samples. Ouabain significantly increased the concentration of camptothecin appearing in the bile. Because some of the agents are themselves choleretic, these data also had to be analyzed in terms of micrograms of camptothecin excreted over 30 minutes and thus probenecid and methotrexate significantly inhibited the rate of excretion of camptothecin into bile. One final method of analyzing these results is to report them in terms of percent of the dose excreted; again it is seen that probenecid, methotrexate, and tyropanoate sodium markedly diminished the amount of drug appearing in the bile.

DISCUSSION

In studying the distribution of camptothecin in mice, it was found that the gastrointestinal tract (plus contents) became the site of concentration of the drug whether the drug was given via the oral or parenteral route. Localization of camptothecin in the gastrointestinal tract persisted about four or five times longer than in any other tissue studied. These data would seem to explain the report of the effectiveness of this agent when administered orally to mice inoculated with L1210 leukemia (1). Since this tumor is usually carried in the peritoneal cavity, it would seem reasonable that the high levels of camptothecin in the area of the intestine would permit the drug to diffuse and exert its action on the leukemic cells in this cavity.

The extensive studies reported here regarding the plasma protein binding of camptothecin are of considerable interest because of the very high degree of binding observed in many species and because we used a variety of techniques. The studies are important because many of the earlier reports have highlighted the reversible nature of the effects of camptothecin on macromolecules such as RNA and DNA (8–15). The reversible nature of the binding of drugs to various proteins found in the plasma has been of interest to pharmacologists for many years (22,23), and it has been generally accepted that only the unbound fraction of a drug possesses the pharmacologic and toxicologic activity of a particular agent. Several reviews (22–26) of both a practical and theoretical nature, have emphasized that the extent to which a drug is bound to plasma proteins may markedly influence its distribution, rate of metabolism, excretion, and interaction with other drugs. Many drugs appear to bind to a single site on plasma proteins, and since camptothecin appears to have this property regarding albumin, the capacity for binding of this drug to a protein is limited. Calculations done by Curry (25) have shown that

small changes in protein binding of drugs in plasma could cause tissue redistribution, and this altered distribution would be expected to be the greatest after changes in tissue binding of highly bound drugs. It also has been pointed out (26) that in drug interactions, especially those involving tightly bound substances, the binding of drugs to plasma proteins has a very important role because of the possibility of adverse drug reactions arising from competition for binding sites. All of the evidence presented here indicates that camptothecin has a very high association constant (7.9×10^6 M^{-1}) and that this drug can be displaced only by drugs of high binding ability, such as dicoumarol. These studies have shown further that for man in particular, about 99% of camptothecin is plasma protein bound. Hence, as has been pointed out for highly bound drugs (26), small changes in binding would be expected to cause relatively large changes in the concentration of free drug, which could lead to more rapid elimination and a redistribution to other tissues. For example, it has been calculated (26) that a decrease in the portion of bound drug from 90% to 80% doubles the plasma concentration of the free drug. Such altered binding of a drug like camptothecin can easily be brought about by the introduction of a second drug, which could compete for the same plasma protein binding site. It is therefore felt that given the very extensive binding which occurs for camptothecin to plasma proteins, one possible explanation for the great discrepancy between the therapeutic efficacy of camptothecin in mice when compared to the poor performance of the agent in man in that there is a tremendous difference in binding in the two species, ie, about 25% (table 3). While this may explain the difference in therapeutic effect, it is difficult to reconcile the toxicity observed (7,19) in monkeys and man since both of these species bind the drug to about the same extent. In the monkey, there was severe gastrointestinal toxicity and leukopenia and mild liver and renal toxicity. Hence it would appear that the reported toxicity in both dogs and monkeys correlated well with the distribution studies in mice that indicated, in particular, localization of the drug in the gastrointestinal tract. The human toxicity reported (7) included nausea, vomiting, diarrhea, and stomatitis; these were predictable from the distribution studies in animals. Since bone marrow was not analyzed for the presence of camptothecin, the effects on this organ site, which were evident in man as leukopenia, thrombocytopenia, and anemia, were not anticipated. On the other hand, it is felt that the plasma drug displacement studies do offer an explanation for the high hematologic toxicity in man. First, as indicated in table 5, drugs which are acids, bases, or neutral molecules, when added in large enough amounts, did significantly alter the binding of camptothecin. Second, it is noted in all three clinical studies (6,7,27) that patients had received or were receiving a variety of drugs along with the camptothecin. Therefore, toxicity might have arisen from similar displacement processes.

The species differences in the plasma protein levels and camptothecin binding (table 3) were of interest from several points of view. The methodology employed here for protein assays included standard clinical methods (16), and these methods must be viewed with caution since they were established primarily for man and may be biased. Nonetheless, the migration properties of the different protein fractions were identical to or very similar to those of man. The values reported here for protein fractions present in most species agree in general with the findings of others (28,29), but not all species values are in full agreement with published surveys (30). The total protein itself, although ranging from 1.7 to 11.0 μg/100 ml, did not correlate very well with the degree of binding of camptothecin. In general, the profile of proteins in man resembled that of proteins in most mammals. On the other hand, because of the apparent importance of the α-globulins in binding camptothecin, the following species had either none or very low levels of either α1- and/or α2-globulins: swine, goat, horse, lamb, bovine, rabbit, mouse, and cat. Table 3 also suggests animal models, lacking certain protein fractions, which could be used to test for specific binding to plasma. For example, there were no detectable levels of either α1- or β-globulins in either the lobster or the dogfish. On the other hand, the albumin-like protein in these two species was pres-

ent at a level approximately equal to that occurring in man. Furthermore, with regard to the dogfish, the large amounts of α2-globulin are of interest. Turtle and frog plasma had no detectable levels of α1-globulin, and, interestingly, the frog had none of the important γ-globulin. Among the avian species studied, only the duck had detectable levels of α-globulin and the general absence of this particular fraction seems to correlate with the low levels of camptothecin binding. The goose plasma analyzed also did not have β- or γ-globulins present. Further study of table 3 should suggest many other interesting possibilities for studying species differences in plasma binding. The use of a rather general statistical tool such as factor analyses did enable us to focus on the fraction which seemed to correlate best, ie, α-globulins, and hence this hint was borne out in the studies of purified protein fractions (table 4). The use of the rather simple device of the filtering cones facilitated studies of the displacement of camptothecin by various drugs. These studies correlated very well in that the most effective drug in displacing camptothecin, dicoumarol, was confirmed to have the same high degree of activity in the fluorescence polarization studies. Since camptothecin has an excellent fluorescence spectrum (4), a study of fluorescence polarization is a very convenient method for measuring the binding of this drug to plasma albumin. These same kinds of studies were also employed to measure drug binding and displacement in plasma samples without prior isolation of the binding proteins. Since many drugs used in the treatment of neoplastic disease are highly fluorescent, this provides a useful alternative method to the classical techniques of dialysis for measuring drug interactions with macromolecules. By the use of this technique, the association constant for camptothecin was determined to be $7.9 \times 10^6\ M^{-1}$ and this value is higher than that reported for dicoumarol (about $1 \times 10^6\ M^{-1}$) (31).

The studies of biliary transport of camptothecin in rat bile confirmed the suggestive evidence that the drug was being excreted by this route in the mouse. The excretion of camptothecin into the bile appeared to be caused, at least in part, by active transport processes. Evidence of this is cited in table 7 where it can be noted that the bile/plasma ratio after 90 minutes was approximately 200; the biliary route dominated even when the renal vessels were not ligated (table 8). The transport of camptothecin (table 10) was significantly inhibited by acids such as probenecid, methotrexate, tyropanoate sodium, and penicillin G, but not by a base (procainamide ethobromide) or a neutral molecule (ouabain). It was further seen from these biliary studies that the rat transport capacity was not saturated very easily even when the doses were increased 1000-fold. This also occurs for methotrexate (32), and hence it is interesting that methotrexate not only inhibited the in vitro binding of camptothecin but also was one of the most potent inhibitors of the biliary transport of camptothecin.

In summary, we have offered pharmacologic evidence strongly suggesting that the gastrointestinal toxicity seen in some species is due to localization of camptothecin at this organ site. It has been shown further that the localization in the gastrointestinal tract is apparently due to rapid excretion of camptothecin into the bile. Species differences in binding of this drug suggest that the therapeutic effectiveness of this agent in the mouse may be due to low plasma protein binding in this species. The lack of therapeutic efficacy of this agent against human neoplasms may be due to either of two points: (*a*) the drug may be rapidly excreted into the bile, and/or (*b*) because of the high degree of plasma binding of this agent, therapeutic levels of free drug may not have been reached to date. The high binding affinity of camptothecin to plasma proteins and the fact that displacement of such drugs may cause significant increases in amount of free drug, points out some of the definite hazards to be considered in this era of multiple drug therapy. The studies of possible inhibitors of binding and biliary transport of camptothecin suggest the possibility of appropriate therapeutic manipulation where the efficacy of this drug may be enhanced by agents which selectively decrease the binding and biliary transport. Of course, structural alterations based on the synthesis of analogs of camptothecin (33–36) may yield compounds of improved therapeutic effectiveness and less toxicity.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Camptothecin: NSC-100880; CAS reg. No. 25387-67-1

Dicoumarol: NSC-17660; CAS reg. No. 66-76-2; coumarin, 3,3′-methylenebis-[4-hydroxy-; Dicumarol

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Ouabain: NSC-25485; CAS reg. No. 630-60-4; ouabain, octahydrate (5β-card-20(22)-enolide, 1β-3β,5,11α,14,19-hexahydroxy-, rhamnoside, octahydrate)

Penicillin G: NSC-402815; CAS reg. No. 7477-78-3

Phenylbutazone: NSC-25134; CAS reg. No. 50-33-9; 3,5-pyrazolidinedione, 4-butyl-1,2-diphenyl-

Probenecid: NSC-18786; CAS reg. No. 57-66-9; benzoic acid, *p*-(dipropylsulfamoyl)-

Procainamide ethobromide: CAS reg. No. 17010-70-7

Tyropanoate sodium: NSC-107434; CAS reg. No. 7246-21-1; hydrocinnamic acid, 3-butyramido-α-ethyl-2,4,6-triiodo-, sodium salt

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A New Antitumor Antimetabolite, (αS,5S)-α -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (NSC-163501): Antimicrobial Reversal Studies and Preliminary Evaluation Against L1210 Mouse Leukemia In Vivo [1,2,3]

Ladislav J. Haňka, David G. Martin, and Gary L. Neil [4,5]

SUMMARY

(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (NSC-163501), a new antitumor antibiotic, was isolated from fermentation liquors of *Streptomyces sviceus*. The activity was discovered in an in vitro screen capable of detecting antimetabolites. Whole lyophilized fermentation liquors were inactive in vivo against L1210 and P388 leukemias in mice. However, activity in vivo was detected with partially purified preparations. Daily intraperitoneal administration of a relatively pure material (about 50% NSC-163501) to L1210 leukemic mice (8 mg/kg given daily for 5 days) yielded a 65% increase in lifespan. Toxicity, as measured by weight change, was minimal at this dose. A microbiological assay for NSC-163501 employing *Bacillus subtilis* has been developed. The assay method is capable of detecting concentrations of 0.1–0.2 μg of NSC-163501 equivalents per milliliter of human plasma. Excellent agreement between microbiological, cell culture, and in vivo assays was observed. The antimicrobial activity of NSC-163501 (about 50% pure) was reversed (antagonized) by histidine and, to a lesser extent, by deoxyinosine.

[Cancer Chemother Rep 57:141–148, 1973]

In 1967, we described a screening system for the in vitro detection of antimetabolite antibiotics (1). This screen was based on the observation that known antimetabolites had a much stronger inhibitory effect on susceptible micro-organisms grown in synthetic media than on those grown in the commonly used complete media (eg, nutrient agar, etc).

Several new agents have been detected in recent years using this screening system. Three antimetabolites of biotin have been found: α-dehydrobiotin (2), α-methyldethiobiotin, and α-methylbiotin (3,4).[6] 2-Amino-4-methyl-4-hexenoic acid, an antimetabolite of leucine (5), was also detected by this screen. Using this technique, we were able to recognize the antimetabolic character of an antibiotic produced by *Streptoverticillium ladakanus* and

[1] Received Dec 19, 1972; revised Apr 5, 1973.

[2] Supported in part by contract PH43-68-1023 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[3] (αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid: U-42, 126.

[4] Cancer Research, The Upjohn Company, Kalamazoo, Mich.

[5] The technical assistance of Miss S. L. Kuentzel, Mrs. A. E. Berger, and Mr. W. E. Titus is acknowledged. The cell culture assays were performed by Mr. H. H. Buskirk.

[6] α-Dehydrobiotin: NSC-127473; 2-pentenoic acid, 5-(*cis*-hexahydro-2-oxo-1*H*-thieno-[3,4-*d*]imidazol-4-yl)-.

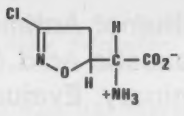
α-Methylbiotin: NSC-145151; 1*H*-thieno[3,4-*d*]imidazole-4-valeric acid, hexahydro-α-methyl-2-oxo-, *cis*-.

later identified as 5-azacytidine (6).[7] This drug was also prepared synthetically by Pískala and Šorm of the Czechoslovak Academy of Sciences (7). On several occasions, we have detected the presence of furanomycin in our fermentations.[8] The discovery of this antimetabolite of isoleucine was also reported by Katagiri et al (8).

Similarly, we have observed that some of our cultures produced *l*-4-oxalysine,[9] an antimetabolite described by Stapley et al (9), or *l*-3(2,5-dihydrophenyl)alanine, an antimetabolite of phenylalanine that was reported by Scannel et al (10).

At the present time, this screen is being utilized by several other laboratories engaged in the search for new antitumor drugs. Among others, Korobkova et al (11, 12) reported some of the results of their screening for antimetabolites at the Academy of Medical Sciences of the USSR, Moscow. Scannel et al (13–16) and Westley et al (17) have described several new antimetabolites detected in recent years at the Hoffmann-LaRoche Research Laboratories. Gordee (18) has reported that some antifungal antibiotics were also more readily detectable in screening systems employing chemically defined cultivation media. A screening program for antimetabolites is being conducted at the Kitasato Institute in Tokyo in the laboratories of Dr. Toju Hata (personal communication, 1972).

Recently, we have discovered another new antimetabolite by using this screen: (αS, 5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (NSC–163501) (fig 1). The production of this drug has been described in a separate communication (19), and the isolation and characterization will be described later. The present communication describes preliminary studies of the antitumor activity of this agent against L1210 mouse leukemia both in vivo and in vitro, the microbiological assay of the agent, and studies of the reversal of its activity in vitro. As a result of our experience in the discovery of this drug, some changes in the procedures employed in determining the antitumor activity of fermentation liquors are proposed.



U-42,126

L-(αS,5S)-α-Amino-3-chloro-2-isoxazoline-5-acetic acid

FIGURE 1.—Chemical structure of NSC-163501.

MATERIALS AND METHODS

Microbiological Assay of NSC-163501 in Human Plasma

The concentrations of NSC–163501 in human plasma were determined by a disc-plate microbiological assay using *Bacillus subtilis* cultivated in a completely synthetic medium (1). Standard solutions were prepared in pooled human plasma at concentrations of 1.0, 0.66, 0.44, and 0.29 μg/ml. The material used in this investigation was about 50% pure. Aliquots (0.08 ml) of standard solutions were applied to filter paper discs (12.7-mm diameter; C. Schleicher and Schuell Co., Keene, NH). The discs were applied in triplicate to the surface of agar trays inoculated with the test organism. The trays were incubated for 18–20 hours at 37 C and the zones of inhibition were measured. A standard curve was constructed and the concentrations of the unknown samples were determined from it. A similar investigation was carried out with solutions of NSC–163501 prepared in a pH 7.0 phosphate buffer.

Reversal (Antagonism) Studies

The first reversal study with NSC–163501 was done at the time of primary in vitro antimicrobial screening. The materials used for testing were round plugs (6-mm diameter) of agar with growth (4 days) of the producing actinomyces on their surfaces. These plugs were applied to the surface of the synthetic agar (control plates) inoculated with a spore suspension of *B. subtilis*. The suspension con-

[7] 5-Azacytidine: NSC-102816; CAS reg. No. 320-67-2; *s*-triazin-2-(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-.

[8] Furanomycin: NSC-116328; CAS reg. No. 15379-21-2; 2-furanacetic acid, α-amino-2, 5-dihydro-5-methyl-.

[9] *l*-4-Oxalysine: NSC-136035; alanine, 3-(2-aminoethoxy)-, dihydrochloride, DL-.

tained about 1.5×10^5 spores per milliliter and the rate of inoculation was 0.5 ml/liter of medium.

In other plates, the synthetic agar was supplemented with varying concentrations of potential reversing agents. The plates were then incubated for 24 hours at 37 C and the zones of inhibition were recorded. Past experience indicates that the stronger the reversing potential of any compound added to the synthetic agar, the smaller the resulting zones of inhibition (as compared to controls). Detailed reversal studies were done later with NSC–163501 which was about 50% pure. In that study, we evaluated the reversing potential of single amino acids and purines.

In Vivo Antitumor Testing

According to the protocols devised by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute (20), lyophilized fermentation liquors were submitted by us to the Illinois Institute of Technology Research Institute (IITRI), Chicago, Illinois, for in vivo evaluation against P388 and L1210 leukemias in mice. The first partially purified preparation (lot 1) was also tested at IITRI and its activity was confirmed in our laboratories. Subsequent preparations (lots 2 and 3) at different stages of purification were tested for activity in vivo and in vitro against L1210 leukemia in our laboratories.

The purity of the various lots was estimated by a microbiological assay against *B. subtilis* cultivated in the synthetic medium (1): lot 1 was about 0.2% pure. This represented an approximately 15-fold purification over the original lyophilized fermentation liquor. Lot 2 was about 3.5% pure and lot 3 was about 50% pure. A sample employed for X-ray crystallographic determination of structure was considered 100% pure.

The parent line of L1210 leukemia was obtained from I. Wodinsky (Arthur D. Little, Inc., Cambridge, Mass) and was maintained by weekly passage (intraperitoneal [ip] inoculation) of ascites cells in female DBA/2 mice (Jackson Memorial Laboratories, Bar Harbor, Me). Therapeutic tests were conducted in female BDF_1 mice (C57BL/6 × DBA/2) from the same source, weighing 19 ± 2 g. L1210 cells (10^5 per mouse) were injected ip on Day 0 and therapy was initiated 24 hours later. Because of the limited quantities of NSC–163501 available, only six mice per treatment group were used. Control (untreated) groups had eight to 12 mice.

Agents were administered ip as solutions in sterile water (0.2-ml volume). Treatment was for 9 consecutive days (Days 1–9). Animals were weighed on Day 1 and again on Days 5 and 9, and average weight changes (g/mouse) were determined. Animals were observed and deaths were recorded twice a day where possible. Percent increases in lifespan (%ILS) were calculated from median and mean survivals and a 25% ILS was considered as significant antitumor activity.

Inhibition of growth of L1210 cells in culture was determined by the method of Buskirk (21). ID50 and ID90 values, concentration (μg/ml) required to inhibit the growth (over a 3-day period) 50% and 90% respectively, were calculated.

RESULTS

Microbiological Assay

A typical standard curve for NSC–163501 in human blood plasma is presented in figure 2. The lowest detectable level for this 50%-pure preparation was about 0.15 μg of NSC–163501 equivalents per milliliter of plasma. Such sensitivity should prove adequate for physiologic disposition studies. The slope of the dose-response curve was rather steep. The sensitivity of the test organism to NSC–163501 was slightly greater when the drug was dissolved in pH 7.0 phosphate buffer than when it was dissolved in human plasma.

Reversal (Antagonism) Studies

The results of the first reversal (antagonism) study made with the agar plugs of the producing micro-organism are presented in table 1. The strongest reversal of the antimicrobial activity was noted with combined amino acids. A partial reversal was noted with combined purines and pyrimidines. The reversal of the antimicrobial activity of 50%-pure NSC–163501 by several single metabolites is pre-

TABLE 1.—Inhibition by NSC-163501 of *B. subtilis* cultivated in synthetic agar further supplemented with different metabolites

Cultivation medium	Diameter of zones of inhibition* (mm)
Nutrient agar	0
Synthetic agar (control)	38
Synthetic agar + amino acid mixture†	0
Synthetic agar + purines-pyrimidines mixture†	28 (very hazy zone)
Synthetic agar + vitamin mixture†	38

*Zero values indicate that no zones were observed around a 12.7-mm disc.

†The composition of all these supplements has been described previously (1).

sented in table 2. Of all the metabolites tested, *l*-histidine was the most effective in preventing the inhibition of *B. subtilis* by NSC-163501. Similar antagonism was also observed with high concentrations of deoxyinosine.

Antitumor Activity of NSC-163501

Lyophilized whole fermentation liquors were inactive against both L1210 and P388 leukemias in mice at the highest nontoxic doses tested: L1210 leukemia, 2% ILS at 200 mg/kg; P388 leukemia, 7% ILS at 400 mg/kg (daily ip doses, 9 days). The first partially purified preparation (lot 1, about 0.2% pure) was tested at IITRI and was reported to be active against L1210 and P388 leukemias (40% and

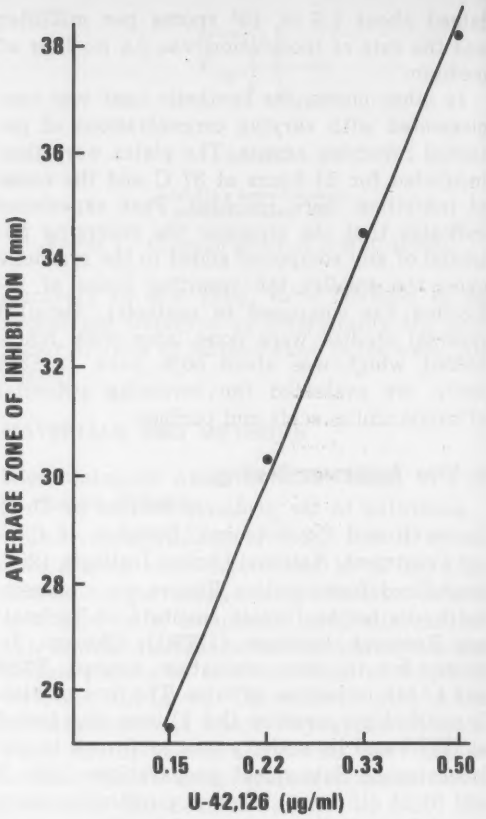


FIGURE 2.—Microbiological assay for NSC-163501 human blood plasma with *B. subtilis*. NSC-163501 equivalent concentrations were based on estimated purity (50%).

TABLE 2.—Inhibition by NSC-163501 (about 50% pure) of *B. subtilis* cultivated in synthetic agar supplemented with several metabolites*

Cultivation medium supplement	Concentrations of NSC-163501 (µg/ml)		
	0.16	0.31	0.62
Control (no supplements)	24	28	35
l-Histidine (5µg/ml)	0	0	0
l-Histidine (2.5 µg/ml)	0	0	trace
l-Histidine (0.5 µg/ml)	trace	18 hazy	29 hazy
Deoxyinosine (100 µg/ml)	trace	21	25

*The numbers in the body of this table are zones of inhibition around a 12.7-mm paper disc. Zero values indicate that no zones were observed around a 12.7-mm disc.

37% ILS respectively, at a daily ip dose of 400 mg/kg, 9 days). Subsequent evaluation of NSC–163501 at various stages of purity was carried out in our laboratories. The results of these studies are presented in table 3.

At a dose of 400 mg/kg/day, we observed a 45% ILS (median) when lot 1 (about 0.2% pure) was used. These results compare favorably with those obtained by IITRI for the same preparation. The best antitumor effects were observed with the 50%-pure preparation (lot 3): the ILS (median) was 65% at a dose of 8 mg/kg/day. Dose responses were observed with all the preparations.

The relative antitumor potencies of the three lots (in vitro and in vivo) are compared in table 4. For preparations ranging from 0.2% to 50% pure, the concentrations (or doses) required to elicit a given antitumor effect, when corrected for this degree of purity, are quite constant. This suggests very strongly that the activities measured with the three bioassays employed (microbiological, cell culture, and L1210 in vivo) are identical. A dose of about 0.5 mg/kg/day (9 days) of pure NSC–163501 would be expected to yield a significant (>25% ILS) therapeutic effect in L1210 leukemic mice.

DISCUSSION

Several interesting observations were made during this study. Whole crude lyophilized fermentation liquors submitted for initial testing were inactive in vivo. Normally, this would lead to suspension of further work on a culture. However, because of the interesting reversal pattern of this particular culture, we decided to test in vivo a partially purified preparation. We noted that a partially purified preparation of rather low purity (lot 1, about 0.2% pure by later analysis) demonstrated significant activity against L1210 leukemia. We have had similar experiences in the past, particularly during work with cultures producing furanomycin, an antimetabolite, the antimicrobial effect of which was reversed most readily by isoleucine (8). The whole lyophilized fermentation liquors of the furanomycin-producing culture were inactive in vivo against Walker 256 carcinosarcoma in rats and L1210 leukemia in mice. However, after partial purification, significant activity against Walker 256 was reported.

Our experience with NSC–163501 and furanomycin-producing cultures can be explained in at least one of the following ways:

TABLE 3.—Antitumor (L1210 leukemia) activity of NSC-163501*

Lot No.	Purity† (%)	Total daily dose‡ (mg/kg/day)	NSC-163501 equivalent dose§	Survival¶ (days)			% ILS**		Average weight change (g/mouse)	
				Range	Median	Mean±SD	Median	Mean	Days 1–5	Days 1–9
		Control	—	8.0–9.0	9.0	8.7±0.5	—	—	+2.3	—
1	0.2	600	1.2	12.5–16.0	14.0	13.3±0.6	55	53	−1.1	−0.3
		400	0.8	11.5–14.5	13.0	13.0±1.0	45	50	−0.7	+0.4
		200	0.4	10.5–13.5	11.0	11.2±1.2	22	29	+0.3	+2.4
2	3.5	30	1.15	10.5–13.5	12.5	12.3±1.2	39	41	−0.2	+1.5
		10	0.35	9.5–11.0	10.5	10.4±0.5	17	20	+0.2	+0.4
		Control	—	8.0–10.5	8.5	8.7±0.8	—	—	+1.6	+3.2
3	50	8	4	9.5–15.5	14.0	14.1±1.0	65	62	+0.2	+2.1
		3	1.5	11.5–14.0	12.5	12.6±1.2	47	45	+0.6	+1.7
		1	0.5	13.0–14.5	10.5	10.6±0.7	24	22	+1.1	+3.8

*Six mice per group (8–12 for controls) were inoculated on Day 0 with 1×10^5 L1210 cells per mouse.
†Purity estimates were based on *B. subtilis* activity.
‡Treatment was ip daily, Days 1–9; agents were administered in 0.2-ml volumes.
§NSC-163501 equivalent dose was based on total dose (partially purified fermentation liquor solids) and estimated percent purity.
¶Mean and standard deviations (SD) were calculated.
**%ILS was calculated from median (or mean) survivals of treated and control groups.

(*a*) The antimetabolite may be produced at such low concentrations in the original fermentation that, although it is detected in the extremely sensitive in vitro screen, it is not of sufficient concentration in the whole lyophilized fermentation liquor to yield significant antitumor activity in vivo where the amount of active drug administered is limited by the "bulk" of inactive material. Commonly, the highest dose employed in the in vivo evaluation of lyophilized fermentation liquors in mice is 600 mg/kg. Thus, in view of the data presented here, it is not surprising that initial results of testing NSC–163501 whole lyophilized fermentation liquors were negative. By microbiological assay, we can estimate in retrospect that such material contained approximately 0.013% NSC–163501. Thus, the actual maximum NSC–163501 dose employed in initial studies was about 0.08 mg/kg, approximately sixfold less than that (0.5 mg/kg) which we now know (table 4) is required to show significant activity in vivo.

(*b*) Although the above explanation may be sufficient to explain the results obtained with NSC–163501, another phenomenon may be at least partially involved. Actinomycetes are routinely cultivated in rich media containing a variety of metabolites potentially able to reverse (in a concentration-dependent manner) the activity of the antimetabolite. Many such metabolites are of course also present in the tissues and body fluids of animals. Conceivably, the total combination of metabolites present in the animal and those accompanying the injected antimetabolite in the crude lyophilized fermentation liquor could result in complete reversal of the antitumor activity in vivo. Partial purification (with resultant removal of contaminating metabolites in the fermentation liquor) could result in a preparation which would yield activity both in vitro (against micro-organisms growing in synthetic media) and in vivo. The evaluation in vivo of partially purified fermentation liquors containing antimetabolite activity (rather than crude whole lyophilized fermentation liquors) would seem to be justified.

The antimicrobial activity of NSC–163501 (about 50% pure) was reversed (antagonized) most strongly by *l*-histidine, and, to a much smaller extent, by deoxyinosine. In order to compare the reversing potential of different metabolites, it is possible to calculate their "reversing indices." The reversing index is defined as the ratio of the minimal concentration of a metabolite necessary to completely reverse the antimicrobial effect of a given concentration of an antimetabolite and that given concentration of the antimetabolite. Obviously,

TABLE 4.—Increase in NSC-163501 potency with purification

Lot No.	Purity* (%)	L1210 cell culture activity† (ID50, μg/ml) Total§	L1210 cell culture activity† (ID50, μg/ml) NSC-163501 equivalent dose¶	L1210 antitumor activity‡ (ED25, mg/kg/day) Total§	L1210 antitumor activity‡ (ED25, mg/kg/day) NSC-163501 equivalent dose¶
1	0.2	ND**	ND**	230	0.46
2	3.5	1.1	0.039	15	0.53
3	50	0.19	0.095	1.0	0.50

*Estimated as described in the Materials and Methods section by microbiological assay using *B. subtilis* growing in synthetic agar as the test organism.

†Inhibition of growth (3-day) of L1210 cells in culture was determined. The ID50 value is the concentration required to inhibit growth by 50%.

‡Data were obtained from a linear regression analysis of data in table 3, plotting log dose against the median %ILS. The ED25 is the dose (mg/kg/day, 9 days) which would result in a 25% ILS.

§Total concentration or dose of partially purified preparations.

¶Calculated from total concentration (or dose) and estimated percent purity.

**ND = not done.

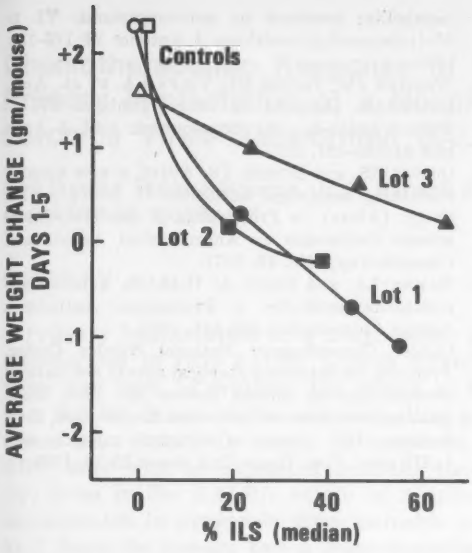


FIGURE 3.—Comparison of therapeutic activity and toxicity (weight change) of NSC-163501 at three stages of purification. Average weight changes (g/mouse) measured between Days 1 and 5 are plotted as a function of the therapeutic effect (% ILS calculated from median survivals) in L1210 leukemic mice for various doses of different lots (of different purity) of NSC-163501.

the smaller the reversing index, the greater the reversing potential of the metabolite. As can be seen in table 2, the addition of 2.5 μg/ml of histidine did prevent the inhibition of *B. subtilis* by NSC–163501 at a concentration between 0.31 and 0.62 μg/ml. Thus, the reversing index of histidine was about $\frac{2.5}{0.5} = 5$.

Toxicity of NSC–163501 at the doses studied can only be estimated based on the limited data (weight change) available. The data from table 3 (average weight change, Days 1–5) are plotted as a function of observed %ILS (median) in figure 3 so that comparisons can be made. It appears that with the most highly purified lot of NSC–163501 (lot 3), there was less observed toxicity (as measured by weight loss) for a given therapeutic effect (%ILS) than was seen for the less pure lots at an equivalent therapeutic effectiveness. Although firm conclusions are not possible because of the limited nature of the toxicity data, the results displayed in figure 3 do suggest that during purification (resulting in lot 3), toxic materials without antitumor activity were removed. In any case, there was no apparent weight loss even at the highest dose of lot 3 (8 mg/kg/day), and it seems likely that higher doses (which would probably result in increased therapeutic effects) would be tolerated. These studies as well as dose scheduling studies, reversal experiments, and investigations of other administration routes must await the preparation of additional quantities of pure NSC–163501.

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Chemotherapeutic Response of Epithelial Tumors Produced by Primary Implantation of a Nonproducer Line of Murine Sarcoma Virus-Transformed BALB/3T3 Cells [1,2]

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SUMMARY

Primary implantation of a nonproducer clone of BALB/3T3 cells transformed in vitro by the Kirsten strain of murine sarcoma virus was used to evaluate the effects of five anticancer agents in a murine tumor model approximating primary carcinoma. The tumor grew relatively slowly in syngeneic BALB/c and hybrid CDF_1 mice with an estimated doubling time in the intraperitoneal (ip) form in the BALB/c strain of 2 days. After subcutaneous (sc) implantation in syngeneic mice, palpable tumors were observed by 7 days. Sc tumors had a mass doubling time of 0.8 days when tumor mass was 100 mg, and 6.3 days when tumors reached a mass of 1 g. Cyclophosphamide proved to be highly effective in prolonging survival of mice inoculated with transformed cells by ip, sc, or intravenous routes. Apparent cures were readily obtained with this agent. BCNU was moderately effective in increasing the survival time of tumor-bearing mice but did not produce long-term survivors. 5-Fluorouracil had a low degree of activity against the ip tumor but did not markedly affect tumor growth or prolong lifespan in mice bearing the sc tumor. Methotrexate and bleomycin had no definite carcinostatic activity against the same tumor induced by the implantation of transformed BALB/3T3 cells. Implications for the use of this transformed cell-induced murine epithelial tumor for the screening and evaluation of drugs useful in the treatment of slow-growing human tumors are discussed.

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Model systems involving transplantable tumors have been used extensively in the screening and evaluation of antitumor agents, and the value of such systems in the selection of

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compounds with activity in clinical neoplasia has been well documented (1–3). Other types of systems, including spontaneous tumors (4–7), first-generation transplants of spontaneous tumors (8–10), primary carcinogen-induced tumors (11, 12), and primary virus-induced tumors (13–17), have also been employed in some measure for the evaluation and detailed investigation of antitumor agents. Although these systems have proven useful, they have, in part due to logistic problems, not been used routinely for the broad-scale screening of antitumor agents.

In the continuing program of investigation of new models for screening and drug evaluation, particularly geared for the development of a system which would select drugs useful in the treatment of human epithelial tumors, it was considered that a nonproducer clone of BALB/3T3 cells transformed in vitro by the Kirsten leukemia pseudotype of murine sarcoma virus (KiMSV) could, upon transplantation into syngeneic mice, provide a chemotherapeutic mold approximating primary carcinoma (18). These cells, when transformed by KiMSV in the absence of murine leukemia virus, have been reported to be free of detectable murine group-specific (gs) viral antigens (19) and have low antigenicity in syngeneic (BALB/c) mice (20). The embryonal-derived BALB/3T3 cell line is carried as a continuous, contact-inhibited, nontumorigenic culture (21). Nonproducer cells transformed in vitro by KiMSV induced progressively growing tumors with histologic features of epithelium in the syngeneic mice (18). These neoplasms behave like spontaneous carcinoma with extensive dissemination and have the capacity to metastasize to the lungs and lymphatic tissue.

In this report we describe the chemotherapeutic activity of cyclophosphamide, methotrexate, 5-fluorouracil (5-FU), BCNU, and bleomycin[a] against primary in vivo transplants of this virus-transformed, nonproducing cell line, using median survival (days) of the recipient mice as an indicator of a chemotherapeutic response.

MATERIALS AND METHODS

Transformed Cell Line

The original line (KA31-clone 234) of KiMSV-transformed, nonproducer BALB/3T3 cells (19) was generously provided by Dr. S. A. Aaronson of the National Cancer Institute (NCI). The cell line was adapted to McCoy's 5A modified medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), and was recloned by microplating (Microtest II, Falcon Plastics, Oxnard, Calif). The recloned line (K-N-8) was frozen in liquid nitrogen and stored prior to use in animal experiments. Aliquots of the frozen cells were thawed and incubated in the medium overnight at 37 C in a 5% CO_2 atmosphere. The cells are mostly spindle-shaped and grow in a criss-cross pattern with a generation time of about 28 hours. Cells for the in vivo inoculations were collected by agitation and were resuspended in McCoy's 5A modified medium without fetal calf serum. Cell concentration was adjusted to the proper inoculum level with a hemocytometer. Cell viability, as determined by staining with nigrosin, was greater than 95%.

Tumor Inoculation

Male BALB/c and CDF_1 (BALB/c female × DBA/2 male) mice, 6 weeks old and weighing 20–25 g, were obtained from the Mammalian Genetics and Animal Production Section, Drug Research and Development (DR&D), Division of Cancer Treatment (DCT), NCI. Transformed cells, suspended in 0.25 ml of medium, were injected by various routes. Subcutaneous (sc) injections were given in the right inguinal region and intravenous (iv) administration was via the tail vein. Animals were housed in groups of eight to 12 per cage and were given Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo) and water ad libitum. Cages were checked daily for deaths.

Tumor Growth Determination

In experiment 9, the growth of local tumors after sc inoculation of transformed cells was determined by measurement of perpendicular

[a] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text. All compounds tested were supplied by DR&D, DCT, NCI.

diameters with vernier calipers at 3–7-day intervals. The growth of the tumors is graphically displayed as the average of the two diameters. For calculation of the mass doubling time of the untreated sc tumor, the tumor mass (assuming unit density) was estimated from the volume formula of a prolate sphere, $4/3\ \pi ab^2$, where a and b were the major and minor semiaxes respectively (10).

Drugs

Cyclophosphamide, 5-FU, and bleomycin were dissolved in normal saline for injection. Methotrexate was dissolved in 2% $NaHCO_3$ and BCNU was dissolved in a minimal volume of 95% ethanol and made up to volume with normal saline for immediate injection. All drug solutions were made up to appropriate concentrations for an injection volume of 1% body weight.

RESULTS

Data in figure 1 for untreated control groups inoculated intraperitoneally (ip) with transformed cells indicate that the lifespan of tumor-bearing mice is a log-linear function of initial tumor load as in some transplanted tumor systems (22,23). BALB/c mice that received 10^6 cells had a median survival time (MST) of 11 days, mice given 10^5 cells had MSTs varying in several experiments from 18 to 25 days (cumulative MST of 20 days), and mice receiving an inoculum of 10^4 cells had an MST of 25 days. Data from the literature (22) for bioassay of ip inoculated L1210 leukemia are shown in figure 1 for comparison. It can be seen that a KiMSV nonproducer-derived tumor is a slower growing tumor than L1210 leukemia, with estimated average doubling times calculated from the slopes of the lines of 55 and 13.5 hours respectively.

The lifespan of mice receiving transformed cells sc was about twice that of mice inoculated ip with a similar number of cells, whereas iv administration of transformed cells resulted in an MST intermediate between the lifespans of mice bearing ip and sc tumors (*see* table 1). Sc tumors were palpable 7 days after implantation of cells and growth of these carcinomas followed Gompertzian growth kinetics (24). The mass doubling times of the untreated sc

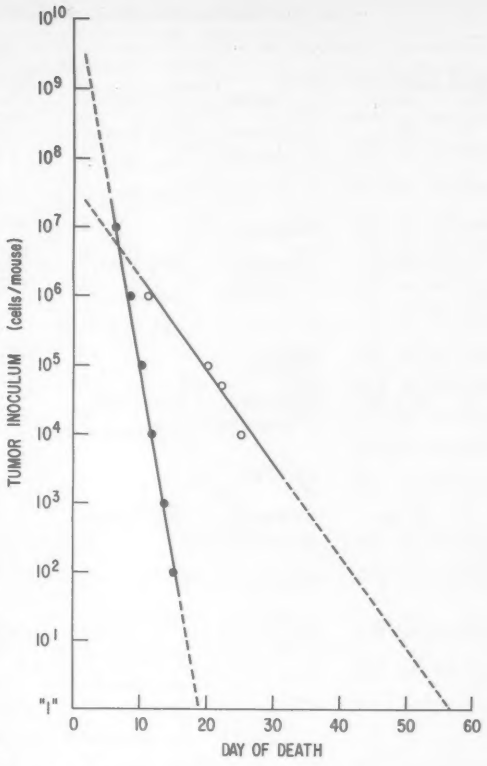

FIGURE 1.—Survival time of mice as a function of ip inoculum level. Open circles (○) represent the survival of mice bearing ip KiMSV-transformed BALB/3T3 cells. The regression line for these points has a correlation coefficient, r = 0.982. Closed circles (●) are bioassay data from the literature (22) for L1210 leukemia, as a comparison.

tumors, calculated from the slope of tangents to the growth curve, were 0.8 days for tumors weighing 100 mg and 6.3 days for tumors weighing 1 g. Of the 162 untreated controls receiving transformed cells by the various routes, there were five mice in which tumors did not develop, for a tumor induction value of 97% (table 1).

KiMSV-transformed BALB/3T3 cells did not proliferate and form tumors in allogeneic C57BL and DBA/2 strains of inbred mice. However, ip implantation of this transformed cell line resulted in tumor growth and death in the

TABLE 1.—Chemotherapeutic response of KiMSV nonproducer tumors

Expt No.	Inoculum,* route	Drug†	Schedule	Daily dosage range (mg/kg)	Optimal dose‡	MST§ (days)	%ILS	Survivors/ total treated¶
1	10^5, ip	Cyclophosphamide	Day 5 only	108–300	108	>200	>900	5/8 at 200 days
			Day 10 only	23–300	180	100	413	3/8 at 200 days
			q4d, Days 5–13	23–65	39	>200	>900	7/8 at 200 days
		Control				19.5		2/16 at 200 days
	10^4, ip	Cyclophosphamide	Day 5 only	108–300	180	>200	>700	7/8 at 200 days
			Day 10 only	23–300	108	>130	>470	4/8 at 200 days
			q4d, Days 5–13	23–65	65	>200	>700	7/8 at 200 days
		Control				25		2/16 at 200 days
2	5×10^4, ip	Cyclophosphamide	Day 6 only	28–132	132	>200	>809	6/8 at 200 days
			q5d, Days 6, 11	220	220	87	295	0/8 at 180 days
		Control				22		0/8 at 25 days
3	10^5, iv	Cyclophosphamide	Day 14 only	65–180	180	78	178	1/8 at 165 days
		Control				28		0/11 at 46 days
4	10^5, ip	Methotrexate	Day 6 only	65–180	65	18	−28	0/8 at 36 days
			q4d, Days 6–18	23–65	23	18.5	−26	0/16 at 24 days
			q4d, Day 6–death	8.4–23	23	22.5	−10	0/8 at 38 days
			qd, Days 6–20	1.08–3.02	1.80	19	−24	0/8 at 24 days
			qd, Day 6–death	0.65–1.80	0.65	21.5	−14	0/8 at 33 days
		Control				25		0/24 at 62 days
5	10^5, ip	Methotrexate	Day 6 only	11–30	18	19	5	1/8 at 140 days
			q4d, Days 6–18	3.88–10.8	3.88	19	5	0/8 at 28 days
			q4d, Day 6 to death	2.33–6.48	2.33	18.5	3	0/8 at 58 days
			qd, Days 6–20	0.23–0.65	0.23	21	16	0/8 at 26 days
			qd, Day 6–death	0.23–0.39	0.23	22	22	0/8 at 35 days
		Cyclophosphamide	Day 6 only	65–180	180	77.5	130	1/8 at 175 days
			q4d, Days 6–18	39–65	39	109	505	3/8 at 175 days
		Control				18		0/24 at 38 days
	10^5, sc	Methotrexate	Day 6 only	10.8–30	30	46	14	0/8 at 71 days
			q4d, Days 6–18	3.88–10.8	10.8	44	9	0/8 at 54 days
		Cyclophosphamide	Day 6 only	65–180	180	67.5	67	1/8 at 175 days
			q4d, Days 6–18	39–65	39	>132	>220	4/8 at 175 days
		Control				40.5		0/8 at 61 days

TABLE 1.—Chemotherapeutic response of KiMSV nonproducer tumors—Continued

Expt No.	Inoculum,* route	Drug†	Schedule	Daily dosage range (mg/kg)	Optimal dose‡	MST§ (days)	%ILS	Survivors/ total treated¶
6	8×10^5, iv	Methotrexate	Day 6 only	65–180	65	21	5	0/8 at 30 days
			q4d, Days 6–18	23–39	39	28	40	0/8 at 30 days
		Control				20		0/11 at 30 days
7	10^5, ip	5-FU	Day 6 only	80–240	160	20.5	14	0/8 at 26 days
			q4d, Days 6–22	40–160	40	22	22	0/8 at 28 days
			q7d, Days 6–20	80–240	80	26	45	0/8 at 41 days
			qd, Days 6–20	10–40	20	26	45	1/8 at 160 days
		Cyclophosphamide	Day 6 only	65–180	180	61	239	1/8 at 160 days
			q4d, Days 6–22	23–65	39	84	367	2/8 at 160 days
			q7d, Days 6–20	65–180	108	112.5	526	3/8 at 160 days
		Control				18		1/12 at 160 days
8	10^6, ip	Cyclophosphamide	q4d, Days 6–18	39–80	80	80.5	632	3/8 at 100 days
		5-FU	q4d, Days 6–18	39–80	39	18.5	68	0/8 at 41 days
		Methotrexate	q4d, Days 6–18	30–60	42	18	64	0/8 at 48 days
		BCNU	q4d, Days 6–18	9.8–20	20	31	181	1/8 at 100 days
		Control				11		0/8 at 46 days
	10^6, ip (CDF_1)	Cyclophosphamide	q4d, Days 6–18	39–80	39	>100	>530	6/8 at 100 days
		5-FU	q4d, Days 6–18	39–80	39	20.5	8	0/8 at 36 days
		Methotrexate	q4d, Days 6–18	30–60	42	18	−6	1/8 at 100 days
		BCNU	q4d, Days 6–18	9.8–20	14	34	79	0/8 at 64 days
		Control				19		0/8 at 33 days
9	2×10^5, sc	Bleomycin	Day 6 only	15–30	15	34	−4	0/12 at 55 days
		Cyclophosphamide	Day 6 only	65–108	65	79.5	124	3/12 at 130 days
			q4d, Days 6–18	23–39	39	86	142	4/12 at 130 days
		5-FU	Day 6 only	40–80	40	34	−4	0/12 at 52 days
			q4d, Days 6–18	10–40	10	34.5	−3	0/12 at 60 days
		BCNU	Day 6 only	10–40	20	36	1	0/12 at 56 days
			q4d, Days 6–18	10–20	10	48	35	0/12 at 76 days
		Control				35.5		0/12 at 55 days

*Transformed cells were inoculated into male BALB/c mice except as indicated in expt 8.
†Drugs were administered sc for ip and iv tumor inoculation and ip for sc tumor inoculation.
‡Dose giving the greatest prolongation of survival.
§Median survival time.
¶Number of treated mice surviving at end of experiment (given as number of days following tumor inoculation); where there were no survivors the day given is the day of death of the last surviving animal in a group.

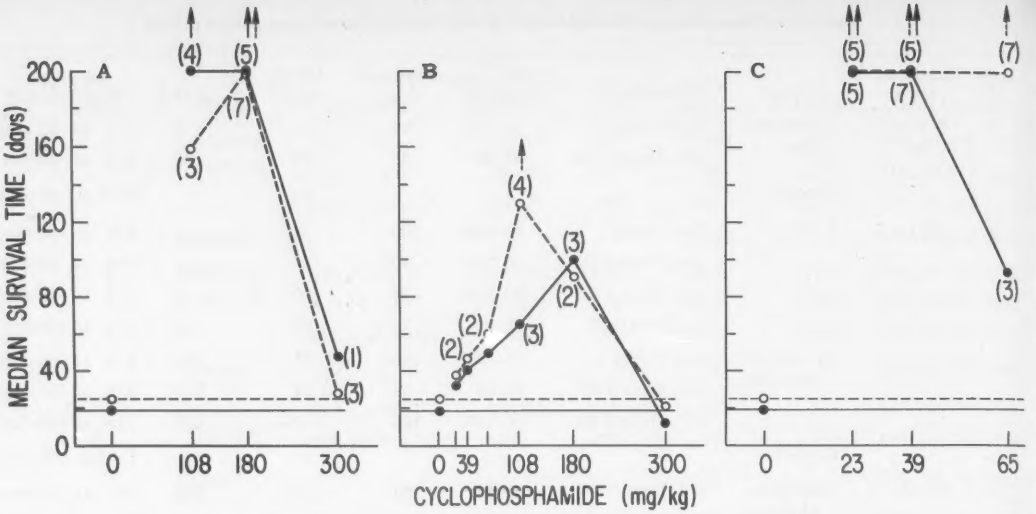

FIGURE 2.—Dose and schedule responses of ip transformed cell-induced carcinoma to cyclophosphamide. Open circles (○) are MSTs of mice inoculated with 10^4 cells; closed circles (●) are MSTs of mice inoculated with 10^6 cells. Numbers in parentheses are long-term survivors out of 8 mice per group. Arrows indicate an MST greater than the point indicated. Lines at 25 and 19.5 days represent MSTs of untreated mice inoculated with 10^6 and 10^4 cells respectively. (*A*) Single sc dose on Day 5 only. (*B*) Single sc dose on Day 10 only. (*C*) q4d, Days 5, 9, and 13.

F_1 hybrid of the syngeneic BALB/c strain with DBA/2 mice (table 1, experiment 8). The lifespan of the CDF_1 mice was 73% longer than that of BALB/c mice in the same experiment.

The results of nine experiments illustrating the in vivo response of the in vitro KiMSV-transformed nonproducer tumors to cyclophosphamide, methotrexate, 5–FU, BCNU, and bleomycin are summarized in table 1.

Cyclophosphamide was highly effective in prolonging the lifespan of tumor-bearing mice and produced long-term survivors with no gross evidence of disease. Sc administration of cyclophosphamide was effective against both ip and iv tumors, while ip treatment with this drug was active against the sc tumor. Cyclophosphamide therapy was highly effective both as a single treatment and on intermittent schedules every 4, 5, or 7 days. A marked therapeutic response was evident against early disease when treatment was started 5 or 6 days after tumor inoculation, when tumors were almost palpable. Since cyclophosphamide treatment gave constant positive chemotherapeutic effects as measured by survival time, it was used as a positive control in the successive experiments.

The survival time-dose relationships for different dosage schedules at two inoculum levels of the ip tumor are shown in figure 2. Cyclophosphamide was effective over a range of doses, particularly on the intermittent dosage schedule where treatment was begun on the 5th day after tumor inoculation. Long-term survivors were obtained in all treatment groups except those which received either toxic or very low doses of the drug on the 10th day after implantation of transformed cells.

The inhibition of local tumor growth at the sc site of inoculation which accompanied the increase in lifespan (ILS) of cyclophosphamide-treated mice is shown in figure 3*A*. With treatment every 4 days (39 mg/kg) local tumor growth was inhibited for an extended period of time. The eight mice in this group of 12 that eventually died had local tumors that had resumed progressive growth 48–60 days after

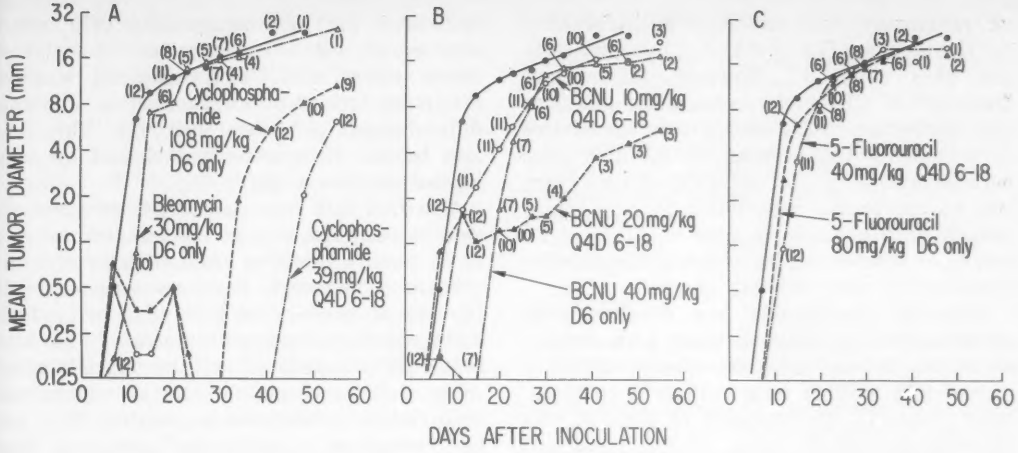

FIGURE 3.—Effect of chemotherapeutic agents on growth of local tumors after sc inoculation of transformed cells. Closed circles (● ——— ●) represent the mean diameter of perpendicular measurement of tumors in surviving untreated mice. All treatment groups consisted of 12 mice. Number of survivors at each time point is given in parentheses. (*A*) Effect of bleomycin and cyclophosphamide on tumor growth. (*B*) Effect of BCNU on tumor growth. (*C*) Effect of 5-FU on tumor growth.

tumor inoculation. Four of the 12 animals were alive at 130 days and were free of palpable local tumors.

After a single injection of cyclophosphamide at 108 or 65 mg/kg (data for the latter dosage level are not shown in fig 3), local tumor growth was inhibited for 3–4 weeks. Thirty-four days after tumor inoculation, progressive local tumor growth was evident in four of 12 mice in both groups. Ten of 12 animals treated at the dosage level of 108 mg/kg and eight of 12 mice treated at the level of 65 mg/kg eventually died with large local tumors. There were one and three survivors in these groups respectively.

Cyclophosphamide was also effective against systemic disease resulting from iv inoculation of KiMSV-transformed cells. A single sc injection as late as 14 days after tumor inoculation produced a definite increase in MST (table 1), although there was only one survivor in the treated group on Day 165 out of eight treated.

The nonproducing character of the K-N-8 cells appeared to be maintained in vivo even after treatment with cyclophosphamide. Five individual sc tumors were excised 1 week after treatment with 10 mg/kg of cyclophosphamide. They were examined by an electron microscope revealing infrequent, nonpathogenic, intracysternal A particles as in the original K-N-8 cells but no type C particles. The homogenates of the tumors contained no infectious MSV virions as determined by focus assays on 3T3FL, NRK, NIH-Swiss 3T3, and BALB/3T3 cells (25). The explants of the tumors showed an identical in vitro morphology to the original K-N-8 cells, and their culture fluid was also negative for infectious MSV.

Methotrexate administered on a single-dose, daily, or intermittent schedule had no reproducible activity against this transformed cell-induced carcinoma (table 1). Definite therapeutic responses (ILS $\geq$ 25%) were obtained in two experimental groups that received methotrexate sc on an intermittent schedule after ip (64% ILS) or iv (40% ILS) inoculation of transformed cells. In other experiments, administration of methotrexate on this schedule (every 4th day) was ineffective in prolonging the lifespan of BALB/c or CDF_1 mice bearing ip or sc tumors.

In two experiments involving ip inoculation

of transformed cells and sc administration of 5-FU, maximum ILS of BALB/c mice was 45% and 68% (table 1). However, intermittent treatment of CDF_1 mice bearing the ip tumor was ineffective. 5-FU also proved ineffective in prolonging the lifespan of BALB/c mice with sc tumors. In this latter experiment there was no appreciable retardation of local tumor growth with a single ip dose of 5-FU at 80 mg/kg or with 40 mg/kg of this antimetabolite administered every 4th day (fig 3*C*).

Although considerably less effective than cyclophosphamide, BCNU, when administered on an intermittent schedule, elicited definitive increases in MST of mice bearing ip or sc tumors (table 1). Prolongation of lifespan was observed in both BALB/c (181% ILS) and CDF_1 (79% ILS) mice with ip tumors. Survival time of mice inoculated sc with transformed cells was not as markedly increased after BCNU therapy (maximum ILS of 35%). However, it can be seen in figure 3*B* that local tumor growth was inhibited by BCNU, particularly at doses which resulted in early death suggestive of drug toxicity (ie, 40 mg/kg, Day 6 only, and 20 mg/kg, q4d, Days 6, 10, 14, and 18).

Bleomycin was tested only on a single-dose schedule against the sc tumor and failed to prolong the lifespan of tumor-bearing mice (table 1). Figure 3*A* illustrates that, even at a toxic dosage level, this antibiotic did not inhibit progressive growth of the local tumor.

DISCUSSION

The primary implantation of in vitro virus-transformed cells in vivo represents a relatively unexplored means of producing experimental tumors for the evaluation of anticancer agents. The development of model tumor systems with distinct histologic, antigenic, and growth characteristics could be anticipated from application of recent studies in the field of in vitro carcinogenesis (ie, 26,27). The tumor arising from the KiMSV-transformed clone of BALB/3T3 cells used in this study possesses several biologic characteristics desirable in a chemotherapeutic model for human cancers, the majority of which are slow-growing carcinomas. The tumor has histologic features of epithelium (18), low antigenicity (20), and a slow growth rate relative to most transplanted tumor systems (28). The transformed clone was originally derived by virus infection of a non-differentiated embryonal cell line. This may also be an etiologic feature shared by some human neoplasms (29).

The fact that infectious MSV does not appear to be propagated in the transformed cells or in tumors resulting from their in vivo implantation, minimizes problems associated with the use of primary in vivo virus-induced tumors as chemotherapeutic models. The high antigenicity associated with tumor cells which carry viral antigens or release viruses can give rise to spontaneous regression (30), and the evaluation of antitumor agents in these systems can be complicated to a greater extent than in tumors with low antigenicity by enhancement of tumor growth due to immunosuppression (31).

Tumor induction by primary implantation of transformed cells is not hampered by the logistic difficulties encountered with the use of primary tumors induced by carcinogens or of spontaneous origin. With these tumor systems, large numbers of animals must be held for extended periods of time and constantly monitored for tumor appearance. Implantation of in vitro transformed cells provides the accurate quantitation and reproducibility of transplanted tumor systems. However, an important drawback of transplanted tumors, the progressive selection on serial transplantation of the most rapidly growing population of tumor cells, is not shared by this system. This system may be free from transplantation antigens.

The chemotherapeutic response of the primary transplants of the KiMSV-transformed nonproducer tumor is generally what one would expect of a slow-growing tumor (28). It was susceptible to treatment with the alkylating agents, cyclophosphamide and BCNU, and the non-cell cycle-specific (32) antimetabolite, 5-FU (table 1). The tumor proved to be refractory to treatment with the S-phase-specific agent, methotrexate (33).

The response of the tumor to the various cytotoxic agents was qualitatively similar after inoculation of transformed cells by different

routes. One exception was the lack of effect of 5-FU on tumor growth (fig 3*C*) or lifespan (table 1) of mice bearing the sc tumor, whereas this drug had moderate activity against the ip form of the tumor (table 1). These data, as well as those showing the more limited prolongation of lifespan of mice with sc tumors after cyclophosphamide or BCNU therapy, suggest that the ip tumor is more sensitive to chemotherapy than the sc tumor.

Our experience with bleomycin was too limited to make a definite determination of activity of this agent against the tumor. This is particularly so in light of the fact that bleomycin was tested only on a schedule utilizing a single dose. Animal (34) and clinical data (35) have indicated that bleomycin is an effective agent in some solid tumors only after prolonged daily administration. It should be noted, however, that a toxic dose of bleomycin had no effect on the growth of the sc tumor (fig 3*A*).

The marked activity of cyclophosphamide against this tumor is interesting in view of the much lesser activity of BCNU. In most experimental tumor systems the latter agent has activity greater than or equivalent to cyclophosphamide. On the other hand, a preliminary tabulation of clinical experience with these and other agents (36) suggests that many of the human solid tumors may be more responsive to treatment with cyclophosphamide than to treatment with BCNU.

These initial studies of the chemotherapeutic response of the KiMSV-transformed carcinoma suggest that this tumor may be a good model system for the selection and evaluation of drugs useful in the treatment of human solid tumors. However, a larger selection of antitumor agents with diverse mechanisms must be screened before the predictability of this tumor model for the various human carcinomas can be determined. These experiments are currently in progress.

In the present systems, conversion from in vitro to in vivo, or from in vivo to in vitro, can be done very easily, which might be beneficial to the study of the mode of action of chemotherapeutic agents and to the in vitro analysis of resistant cell population.

LIST OF COMPOUNDS

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Bleomycin: NSC-125066

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl] methylamino]benzoyl]-; Amethopterin

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Clinical Experience With Two New Methanesulfonic Acid Esters of Aminoglycols, NSC-140115 and NSC-140117, in the Treatment of Malignant Lymphoma [1,2]

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SUMMARY

Twenty-three patients with stage IVA or IVB malignant lymphoma who had never received any chemotherapy or radiotherapy were treated every other day with intravenously administered injections of either 50 mg of 4,4′-biphenyldisulfonic acid compounded with 3,3′-(methylamino)di-1-propanol, dimethanesulfonate (ester) (1:1) (NSC-140115) or 100 mg of 3,3′-iminodi-1-propanol, dimethanesulfonate (ester), *p*-toluenesulfonate (NSC-140117) for a total dose of 1500 mg; no maintenance therapy was given. Six of the 13 patients (46%) treated with NSC-140115 had complete responses and four (30%) had good responses (at least 50% reduction in tumor size). Two of ten patients treated with NSC-140117 had complete responses which have been maintained for 1 year and six had good responses. No toxic effects in the form of skin reactions or gastrointestinal or neurologic complications were encountered, and the drugs were well tolerated by all the patients. These results proved the promising role of NSC-140115 and NSC-140117 in the treatment of malignant lymphoma and encourage further studies of this drug in the treatment of different neoplasms.

[Cancer Chemother Rep 57:159–164, 1973]

In a previous report,[5] we have discussed the effect of NSC-140117 in the treatment of chronic myeloid leukemia. In this report, we present a summary of our clinical experience with NSC-140115 and NSC-140117 in the treatment of malignant lymphomas. The chemistry and screening data for both drugs have been reported by El-Merzabani and Sakurai (1–3).

MATERIALS AND METHODS

Twenty-three patients with malignant lymphoma were studied. Diagnosis was reached by clinical, hematologic, radiologic, and pathologic examinations.

Clinical examination verified glandular, splenic, and hepatic involvement, as well as skin manifestation and the presence or absence of

[1] Received Jan 17, 1973; revised Apr 4, 1973.

[2] NSC-140115: 4,4′-biphenyldisulfonic acid compounded with 3,3′-(methylimino)di-1-propanol, dimethanesulfonate (ester) (1:1); No. 838D.

NSC-140117: CAS reg. No. 32784-82-0; 1-propanol, 3,3′-iminodi-, dimethanesulfonate (ester), *p*-toluenesulfonate; No. 864T.

Clinical samples of the drugs were kindly supplied by Yoshitomi Pharmaceutical Ind., Ltd., Osaka, Japan.

[3] Cancer Institute, Cairo University, Cairo, Egypt.

[4] Cancer Institute, Tokyo, Japan.

[5] Gad-el-Mawla N, Abul-Enien M, Hamza MR, et al. Evaluation of 3,3′-imino-1-propanol, dimethane-sulfonate (ester), *p*-toluenesulfonate No. 864T (NSC: 140117) in the treatment of chronic myeloid leukemia. Submitted for publication.

systemic manifestations such as fever, loss of weight, pallor, and sweating.

Radiologic examination included X-ray films of the chest, lung parenchyma, and pleura. Lymphangiography was done for detection of involvement of abdominal glands. Patients were given barium meals for studies of the small intestine when gastrointestinal lymphoma was suspected.

Hematologic examination including determination of values for the peripheral blood and sternal marrow puncture was done for all patients.

Pathologic examination was done on glands biopsied for diagnosis.

Performance scores (Karnofsky scale) were recorded.

All patients had stage IVA or IVB disease according to the Rye classification (4). No patient had received any chemotherapy or radiotherapy prior to the study.

NSC-140115 was given to 13 patients with malignant lymphoma (table 1). Eleven of the patients were male and two were female. Their ages ranged from 9 to 65 years. Four patients had reticulum cell sarcoma, four patients had lymphosarcoma, and five patients had Hodgkin's disease. NSC-140115 was given by intravenous (iv) injection at a dose of 50 mg every other day for a total dose of 1500 mg. No maintenance therapy was given.

NSC-140117 was given to ten patients with malignant lymphoma. Six of the patients were male and four were female. Their ages ranged from 18 to 50 years. Five patients had reticulum cell sarcoma, three patients had Hodgkin's disease, one patient had lymphosarcoma, and one patient had multiple myelomatosis (table 2). NSC-140117 was given by iv injection at a dose of 100 mg every other day for a total dose of 1500 mg. No maintenance therapy was given.

All patients were followed serially, clinically for the determination of glandular enlargement and liver and spleen size, radiologically, and hematologically. Hemoglobin values, red blood cell (rbc) counts, white blood cell (wbc) counts (total and differential), and platelet counts were determined weekly during the period of drug administration and every 2 weeks

TABLE 1.—Clinical results of NSC-140115 treatment in patients with malignant lymphoma

Patient No.	Age	Sex	Diagnosis	Involvement* of—			Response†
				Glands	Liver	Spleen	
1	25	M	Lymphosarcoma	++++	++	++	Good
2	35	M	Reticulum cell sarcoma	++++	++	+++	Complete
3	50	M	Hodgkin's disease	+++	++	++	Complete
4	26	M	Lymphosarcoma	+++	++	++	Complete
5	42	M	Lymphosarcoma	++++	+++	+++	Good
6	65	M	Reticulum cell sarcoma	+++	++	++	Partial
7	43	M	Hodgkin's disease	+++	++	++	Complete
8	21	F	Hodgkin's disease	++++	++	++	Good
9	9	M	Reticulum cell sarcoma	+++	++	++	Partial
10	12	M	Hodgkin's disease	+++	++	++	No response
11	23	F	Lymphosarcoma	+++	++	++	Good
12	40	M	Hodgkin's disease	++++	++	++	Complete
13	18	M	Reticulum cell sarcoma	+++	++	++	Complete

*Extent of involvement: Glands: +++ = involvement of cervical, axillary, and inguinal groups of glands; ++++ = involvement of cervical, axillary, inguinal, and mediastinal groups of glands. Liver: ++ = 2 cm below costal margin; +++ = 3 cm below costal margin. Spleen: ++ = midway between costal margin and umbilicus; +++ = reaching to the umbilicus.

†*See* text for explanation of response ratings.

TABLE 2.—Clinical results of NSC-140117 treatment in patients with malignant lymphoma*

Patient No.	Age	Sex	Diagnosis	Involvement of—			Response
				Glands	Liver	Spleen	
14	35	M	Reticulum cell sarcoma	++++	++	++	Good
15	50	M	Reticulum cell sarcoma	+++	++	+	Complete
16	30	F	Hodgkin's disease	+++	+	+	Good
17	80	F	Hodgkin's disease	+++	++	++	No response
18	30	F	Hodgkin's disease	++++	++	++	Good
19	18	M	Reticulum cell sarcoma	Mycosis	fungoides		No response
20	55	F	Lymphosarcoma	++++	+	+	Good
21	29	M	Multiple myelomatosis	−	−	−	Good
22	30	M	Reticulum cell sarcoma	+++	++	+	Good
23	32	M	Reticulum cell sarcoma	+++	+	+	Complete

*See footnotes for table 1 and text for explanation of organ involvement and response ratings.

during the observation period. A drop in the wbc to less than 3000 cells/mm³ or in platelet count to less than 100,000 platelets/mm³ required temporary cessation of the treatment. Treatment was resumed when the counts returned to these levels. No other therapy that could have an effect on the disease was given concomitantly. The enlarged glands and the liver and the spleen were measured weekly. Measurements were made the same way each time and by the same observer. Serial chest X-ray films were repeated monthly for the evaluation of the intrathoracic lesions. The Wilcoxon T test was used to detect the significance of blood cell count changes.

RESULTS

The effect of treatment was determined at the end of the study period in accordance with the following criteria:

(*a*) Complete response: Complete disappearance of all objective evidence of the tumor in all of the sites being measured.

(*b*) Good response: A decrease of 50% or more of the involved regions.

(*c*) Partial response: A decrease of less than 50% of the involved regions.

(*d*) No response: Progression of tumor growth.

NSC-140115 Treatment

Complete responses were encountered in six of the 13 patients (nearly 46%). In these patients, all glandular enlargement completely disappeared, and the liver and spleen returned to normal size. Resolution of all intrathoracic lesions was also noted. The diseases of four of the six patients are still in remission 10 months after treatment. One patient (No. 3) who had nasopharyngeal involvement before treatment had a relapse after 8 months.

Good response was achieved in four patients (nearly 30%). In these patients, remission lasted up to 6 months and then relapse occurred.

Partial response was achieved in two patients; however, they had relapses immediately after the therapy was discontinued.

No toxic effects in the form of skin, gastrointestinal, or neurologic manifestations were encountered. The drug was well tolerated by all patients. Patient No. 8 had jaundice during intake of the drug. A liver function test showed an increase in alkaline phosphatase (22 King-Armstrong units) with normal serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase values. Accordingly, treatment was continued on the assumption that the jaundice was due to hepatic lymphoma and not to drug toxicity. The jaundice gradually disappeared.

TABLE 3.—Hematologic values of patients before and after treatment with NSC-140115

Patient No.	Hemoglobin (g/100 ml)		Rbc ($\times 10^3$ cells/mm^3)		Total leukocytes (cells/mm^3)		Neutrophils (cells/mm^3)		Platelets ($\times 10^3$ platelets/mm^3)	
	Before	After	Before	After	Before	After	Before	After	Before	After
1	11.5	7.4	3680	2190	4830	500	3467	80	115.0	10.0
2	11.5	9.0	3660	2740	3700	1400	2201	1162	58.5	11.9
3	13.0	8.1	4430	3530	8290	500	5637	80	56.7	20.0
4	12.0	8.7	4050	2440	23400	2750	6318	1815	150.0	94.0
5	14.5	12.0	4700	3110	4500	5200	3060	3536	73.2	10.0
6	10.8	10.5	3990	3670	13400	4200	10522	3318	293.1	51.5
7	7.8	10.1	2420	3930	8300	3550	5140	2130	300.0	35.4
8	9.9	7.8	3110	2430	5700	2100	4845	1617	300.0	18.4
9	8.9	8.7	3190	3680	10400	600	9125	348	300.0	36.0
10	12.5	9.9	3900	3220	10450	1850	8360	1591	400.0	21.2
11	7.8	8.3	3020	3120	14850	3100	3007	2542	468.0	102.3
12	6.3	7.5	2649	2670	17100	1200	13338	804	284.0	25.0
13	13.5	6.9	4260	3010	7900	2900	4503	1769	354.4	50.4
Mean values	10.7	8.8	3634	3057	10447	2312	6118	1599	250.4	36.5
Wilcoxon test	Significant ($P = 5\%$)		Not significant		Significant ($P < 1\%$)		Significant ($P < 1\%$)		Significant ($P < 1\%$)	

A significant decrease ($P < 5\%$) in the mean hemoglobin value was noted (table 3). In addition, there was a marked significant drop in the mean total leukocyte count (mainly in the neutrophil count [$P < 1\%$]) and a significant reduction in the mean platelet count ($P < 1\%$). The drop in the mean rbc was not statistically significant.

Although the thrombocytopenia was severe, only one patient (No. 8) had a bleeding tendency; this patient had jaundice as well. Leukopenia was marked in patient No. 2 (table 1): the total wbc was 1400 cells/mm^3 soon after the cessation of treatment with NSC-140115. This patient had a furuncle on his face, and appropriate antibiotics were given, but jaundice developed within 2 days, and the patient passed into a coma and died within 2 days. Postmortem examination revealed white necrotic areas in the portal tracts of the liver and the splenic pulp. There was no obstruction of the biliary flow as revealed by the flow of the bile on compression of the gall bladder. Microscopic examination revealed septic hepatitis and marked depletion of the lymphoid elements in the spleen and lymph nodes with no residual malignancy. Clumps of bacteria were present in the sinuses of the spleen. Toxic nephritis and myocarditis with fragmentation of the cardiac muscles were evident.

NSC-140117 Treatment

Complete response was achieved in two patients; these patients are still in remission 12 months after treatment. Good response was achieved in six patients; however, they had relapses in 3–4 months. There was no response in two patients. No side effects were observed, and the drug was well tolerated by all the patients.

The decreases in the mean hemoglobin value and the mean rbc (table 4) were not significant. The drop in the mean total leukocyte count (mainly affecting the neutrophils) was significant ($P < 1\%$); however, the drop in the mean platelet count was not significant.

DISCUSSION

Chemotherapy for malignant lymphoma in the late stages (stage IIIB and stage IV) has been attempted in the last 30 years. Up to 1963 single drugs were used; subsequently, combina-

TABLE 4.—Hematologic values of patients before and after treatment with NSC-140117

Patient No.	Hemoglobin (g/100 ml)		Rbc ($\times 10^3$ cells/mm^3)		Total leukocytes (cells/mm^3)		Neutrophils (cells/mm^3)		Platelets ($\times 10^3$ platelets/mm^3)	
	Before	After	Before	After	Before	After	Before	After	Before	After
14	8.0	7.0	2430	2210	6250	1200	3438	840	220.0	198.9
15	8.5	10.0	2660	3540	3600	3900	1800	1316	210.8	212.4
16	13.5	8.5	4360	2900	6600	3000	4488	2160	280.0	180.0
17	10.5	8.5	3540	2250	9000	3700	5760	3145	190.0	67.5
18	7.0	5.5	2360	2340	13620	9350	10360	6545	460.0	245.0
19	8.0	7.0	2220	2240	5600	3750	3024	1913	275.0	210.8
20	9.0	12.0	2650	3740	20900	10100	18183	6767	250.0	336.6
21	12.0	12.0	3630	3090	2900	4000	1363	3200	190.0	234.0
22	13.5	14.5	3960	4230	17000	13600	14960	12240	220.0	200.0
23	13	—	3750	—	4000	—	3120	—	—	—
Mean values	10.0	9.3	3167	2940	9496	6955	7042	4236		
Wilcoxon test	Not significant		Not significant		Significant ($P < 1\%$)		Significant ($P < 1\%$)		Not significant	

tion chemotherapy was tried. Four classes of anticancer agents (each with significant and independent anticancer effect) were tried: alkylating agents; vinca alkaloids; methylhydrazine derivatives (eg, procarbazine); and corticosteroids.[6] Ultmann and Nixon (5) collected the results of studies with these drugs. The response rate was 61% with cyclophosphamide, 64% with vinblastine, and 75% with procarbazine.[7] A response was defined as a 50% reduction in the tumor lasting longer than 2 months. In most studies, the rate of complete response for any of the drugs mentioned previously rarely exceeds 20% (6). The average duration of response to single agents is short (approximately 6 weeks) and appears to be related to the magnitude of reduction in tumor size since the average duration of a complete remission after cessation of treatment is twice as long as that of a partial response. Maintenance of the remission with continued administration of the drug can prolong the response to 8 months for patients having good initial response to drugs.

In the present study, six (46%) of the 13 patients treated with NSC-140115 had complete responses and four (30%) had good responses (at least a 50% reduction in the tumor). Thus, 76% of the patients had responses. Moreover, complete response has lasted for 10 months and perhaps more in four of the six patients treated with this drug. A good response was maintained for up to 6 months in four patients.

Complete response has been maintained for 1 year in two of the ten patients treated with NSC-140117 and good response was achieved in another six patients. Thus, 80% of the patients treated with NSC-140117 responded.

Thus, the results obtained in this study prove the promising role of both NSC-140115 and NSC-140117 in the management of malignant lymphoma. This encourages further large-scale trials with maintenance therapy.

Rats have been protected to some extent against the leukopenia induced by both drugs (particularly NSC-140115) by treatment with 2-mercaptopropionylglycine without affecting antitumor activity (7). The addition of a similar compound to clinical preparations of both drugs might minimize such hematologic toxicity in man.

[6] Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride.

[7] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate.

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Delayed Toxicity of 4′-Demethylepipodophyllotoxin 9-(4,6-*O*-2-Thenylidene-β-D-glucopyranoside) (NSC-122819; VM-26) in Mice [1,2]

Thomas L. Avery, DeWayne Roberts, and Robert A. Price [3,4]

SUMMARY

The podophyllum derivative, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside) (NSC-122819; VM-26), was tested against the transplantable murine leukemias, L1210 and L5178Y. At doses of 2.2, 3.6, 6.0, 10.0, and 16.0 mg/kg, the drug eradicated the tumor in up to 90% of BDF_1 mice inoculated with 10^6 L1210 cells. The survival time of mice succumbing to L1210 leukemia was extended by 94%–173% on the average. Even greater oncolytic efficacy was observed for L5178Y leukemia. A cure rate of 100% was obtained with all but the 2.2-mg/kg dose. At that dose level, 20% of the mice were cured, and the survival time of the remaining mice was extended by 171%. In mice cured of tumor, an unexpected, delayed toxicity developed; this resulted in 100% host deaths at the 16.0- and 10.0-mg/kg doses and a high incidence of fatality at the 6.0-mg/kg dose. Within the limits of 6.0–16.0 mg/kg, each milligram-per-kilogram increase in NSC-122819 dose shortened the mean survival time of non-tumor-bearing mice by approximately 1 week. Delayed toxicity developed in mice of six strains. A statistically significant variation in toxic response was observed among the different strains. The pathologic lesion responsible for delayed toxicity and eventual death was not identified, but evidence of chemical peritonitis tentatively linked development of delayed toxicity to the route (intraperitoneal [ip]) of drug administration. Late toxic deaths did not result from ip administration of small but therapeutically effective doses of NSC-122819 and did not follow intramuscular or subcutaneous administration.

[Cancer Chemother Rep 57:165–173, 1973]

Podophyllotoxin from the rhizomes and roots of the plant *Podophyllum peltatum* is the starting material for the synthesis of derivatives that are classified collectively as podophyllum compounds (1–3). The podophyllums

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[2] Supported by research grants CA-11148, CA-12732, and CA-08480 from the National Cancer Institute, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, and by American-Lebanese-Syrian Associated Charities (ALSAC).

[3] Laboratories of Pharmacology (Drs. Avery and Roberts) and Pathology (Dr. Price), St. Jude Children's Research Hospital, Memphis, Tenn.

[4] The authors thank Sandoz Pharmaceuticals for making NSC-122819 available for this study, Dr. William Sterling for providing results of ongoing studies, and Drs. Lenore Englander and Warren Johnson for their assistance with the pathologic studies.

were effectively used in the treatment of condylomata acuminata (4) and solid tumors (5, 6). Generally, their action resembles that of colchicine with mitotic arrest in metaphase (1,2,7–9).

The podophyllum 4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside) (NSC-122819; VM-26) induced metaphase arrest (10); but, unlike previously described podophyllums (11), this compound also inhibited thymidine incorporation into DNA and prevented entry of cells into prophase (10). In addition, NSC-122819 increased the lifespan of mice inoculated with transplantable tumors (10).

In the treatment of neoplastic diseases, drugs with different therapeutic modalities are frequently combined. We considered NSC-122819, with its apparent bimodal therapeutic action, to be of potential usefulness in combination therapy with other oncolytic agents, and we started preclinical testing with the transplantable murine leukemias, L1210 and L5178Y. In these studies, an unexpected, delayed toxicity was observed. A preliminary report of these data has been presented (12).

MATERIALS AND METHODS

Female mice weighing approximately 18 g were housed in a central animal facility under conditions of uniform caging, 78 F temperature, 55% relative humidity, food and water ad libitum, and 12-hour photoperiods with the light phase commencing at 6:00 AM. The BDF_1, CDF_1, DBA_2, C3H, A, and C57BL strains of mice from Jackson Laboratories, Bar Harbor, Maine, were used, but, unless stated otherwise, the term "mice" will refer to BDF_1 strain. The mice were inoculated intraperitoneally (ip) with 10^6 L1210 or L5178Y ascites tumor cells.

Treatment was started on the day after tumor cell inoculation (Day 1) and repeated on the fourth and seventh days. This schedule appeared to be almost optimal for NSC-122819 (10) and permitted comparison with our studies of other oncolytic drugs. NSC-122819 was administered in volumes of 0.01 ml/g of body weight to provide doses ranging from 2.2–16.0 mg/kg of body weight. Sixteen mg of NSC-122819 was dissolved in 0.1 ml of dimethyl sulfoxide (DMSO), to which 1 ml of Tween-80 and 0.9% NaCl to a final 10-ml volume were added in order. Vehicle without drug was administered to control animals. As an additional control, 5 mg of NSC-122819 in 60 mg of *N,N*-dimethyl acetamide, 500 mg of cremophor EL (polyethoxylated ricinus oil), and ethanol to 1 ml (preformulated by Sandoz Pharmaceuticals) was diluted in 0.9% NaCl for study. Unless specifically stated otherwise, the drug was administered ip.

Drug effectiveness was evaluated by the lifespan of treated tumor-bearing hosts relative to control animals. Mice that lived 45 days after inoculation and at postmortem examination lacked organomegaly, ascites tumor cells, or nodular tumors in the mesentery, pancreas, inguinal and ovarian fat pads, and inoculation site were considered cured, although some animals were observed for 400 days. Composite data are expressed as the mean ± one standard deviation.

Drug effects on the host were assessed on the basis of weight changes, appearance, activity, and postmortem examination. Death was attributed to acute toxicity when mice died earlier than those treated with lower doses, before control mice, or two standard deviations earlier than treatment mates, without evidence of tumor growth or organomegaly. For histologic evaluation, the liver, pancreas, intestine, thymus, marrow, kidneys, spleen, and lungs from BDF_1 female mice not inoculated with tumor were collected, weighed, preserved in 10% buffered formalin, embedded in paraplast, cut into 5μ sections, and stained with hematoxylin and eosin.

RESULTS

Tumor Response

At various dose levels NSC-122819 eradicated L1210 or L5178Y leukemia. The incidence of cure increased from 10% with 2.2 mg/kg to 95% with 16.0 mg/kg. These values reflect composite data from two studies with each tumor line. NSC-122819 was oncolytically more effective against L5178Y leukemia than against L1210 leukemia (table 1). At doses of 6.0, 10.0, and 16.0 mg/kg, NSC-122819 pro-

TABLE 1.—Response to NSC-122819 of BDF_1 mice inoculated with tumor*

Dose (mg/kg)	Tumor line	Tumor response				Death from delayed toxicity		
		No. of mice	Mean survival time† (days)	Increase in lifespan (%)	Cures (%)	No. of mice	Lifespan† (days)	No. of survivors
Saline	L1210	10	6.4 ± 0.3					0
	L5178Y	10	11.9 ± 1.2					0
Vehicle	L1210	10	6.4 ± 0.4					0
	L5178Y	10	12.3 ± 1.3					0
	None (control)	—						5
16.0	L1210	1	13	103	90	9	95.9 ± 28.8	0
	L5178Y	0			100	10	100.8 ± 38.9	0
	None (control)	—				5	38.2 ± 5.2	0
10.0	L1210	4	17.3 ± 1.0	170	60	6	74.2 ± 17.2	0
	L5178Y	0			100	10	66.7 ± 13.3	0
	None (control)	—				5	80.4 ± 20.0	0
6.0	L1210	6	17.5 ± 4.0	173	40	4	99.8 ± 4.0	0
	L5178Y	0			100	8	124.6 ± 60.9	2
	None (control)	—				4	112.0 ± 13.0	1
3.6	L1210	10	16.6 ± 2.7	159	0	0		0
	L5178Y	0			100	0		10
	None (control)	—				0		5
2.2	L1210	10	12.4 ± 2.4	94	0	0		0
	L5178Y	8	33.3 ± 7.7	171	20	0		2
	None (control)	—				0		5

*Composite data from two studies with each tumor line. Ten animals with each tumor were in each treatment group. Data for animals succumbing to the tumor and for those dying of delayed drug toxicity were analyzed separately.

†Mean ± 1 SD.

duced cures in 90% of L1210-leukemia-bearing hosts and extended the lifespan of mice succumbing to tumor by as much as 173% over that of control animals. At doses of 2.2 and 3.6 mg/kg, no mice were cured, but lifespan was increased by 94% and 159%, respectively. At a dose of 2.2 mg/kg, the mean lifespan of mice that succumbed to L5178Y leukemia was extended by 171%, and 20% of the mice were cured. All of the L5178Y-leukemia-bearing mice treated with NSC-122819 at a dose of 3.6 mg/kg or more were cured. The lifespan of tumor-bearing mice treated with vehicle was identical to that of control mice treated with saline.

Host Response

In the preceding studies, acute, drug-induced toxicity was not observed. Mice treated with NSC-122819 remained alert and generally free of morbidity. However, in mice cured of tumor, an unexpected, delayed, and fatal toxicity developed (table 1). Nineteen mice cured of L1210 leukemia died with a mean lifespan of 90 days after tumor inoculation. Twenty-eight of 30 mice cured of L5178Y leukemia died with an average posttreatment lifespan of 96 days.

The incidence of delayed fatal toxicity was related to dose. Late deaths were not observed after treatment with 2.2- or 3.6-mg/kg doses of NSC-122819. Eight of ten mice treated with 6.0 mg/kg died, and all mice treated with 10.0 or 16.0 mg/kg died. For tumor-bearing mice, the earliest death from drug toxicity occurred 50 days after tumor cell inoculation and the latest death occurred 274 days after tumor cell inoculation.

Delayed deaths were also observed in uninoculated control mice. In general, the pattern of these delayed deaths paralleled that in mice inoculated with tumor cells. No deaths occurred with NSC-122819 doses of 2.2 or 3.6 mg/kg.

Four of five mice treated with 6.0 mg/kg and all mice treated with 10.0 or 16.0 mg/kg died. Two differences were observed between the delayed toxic responses of mice inoculated with tumor cells and mice not inoculated with tumor cells. First, at a dose of 16.0 mg/kg, mice lacking tumor died much earlier than mice with tumor. The average lifespan of non-tumor-bearing mice was 38 days compared with roughly 100 days for tumor-bearing mice. Second, variation in time of death was more closely related to drug dose for non-tumor-bearing mice than for tumor-bearing mice. For mice not inoculated with tumor, the relationship between dose and time of death is expressed by the regression equation, $Y = 154.7 - 7.3X$, in which Y represents survival time in days and X represents NSC-122819 dose in milligrams per kilogram. Within the limits of 6.0–16.0 mg/kg, each milligram-per-kilogram increase in dose reduced survival time by approximately 1 week. Late deaths did not occur in non-tumor-bearing mice treated with vehicle.

The first evidence of delayed abnormality was distention of the abdomen due to ascites and megacolon (fig 1). This condition progressed, and lethargy and ruffled hair provided evidence of increasing morbidity. Intestinal dilatation was most pronounced in the proximal colon and cecum, less pronounced in the distal colon and ileum, and occasionally present in other segments of the small intestine. Ascitic volumes of 9 ml were observed with a mean of 3.4 ± 2.6 ml for animals in an advanced stage of toxic reaction. Although sparsely populated with cells, the ascitic fluid contained monocytes, polymorphonuclear leukocytes, plasma cells, mesothelial cells, and red blood cells. Removal of fluid from these mice resulted in body weights that were approximately 15% less than those of control animals. The absence of inguinal and ovarian fat pads and depletion of mesenteric fat added to the appearance of debilitation.

Liver weight was approximately 25% below normal weight ($P<0.01$); occasionally it was reduced to less than half of the normal weight. The liver was morphologically atypical with condensed, rounded lobes which suggested preceding necrosis (fig 2). A thickened fibrous capsule and marginal areas of necrotic parenchymal cells were observed (fig 3). However, massive hepatic necrosis and fibrotic replacement were not visible at histologic examination. Parenchymal cells were generally characterized by reduced cytoplasmic volume and nuclei with pronounced variation in size and chromatin content.

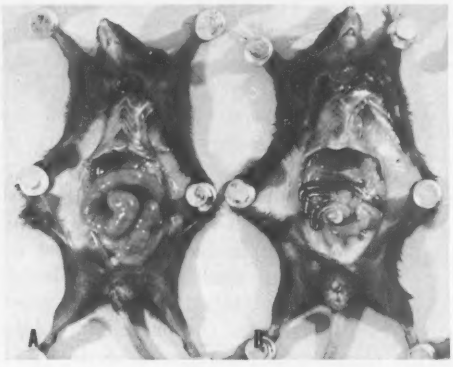


FIGURE 1.—*A*. Abdominal viscera of a non-tumor-bearing BDF_1 mouse treated with NSC-122819 showing the megacolon and rounded liver borders which are characteristic of delayed toxicity. *B*. Abdominal viscera of a normal mouse.

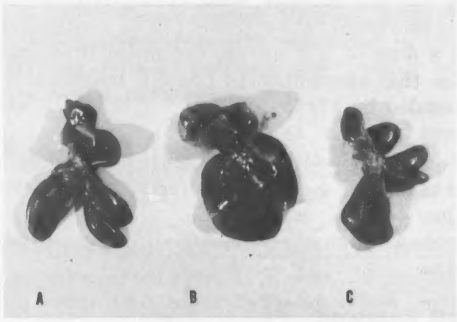
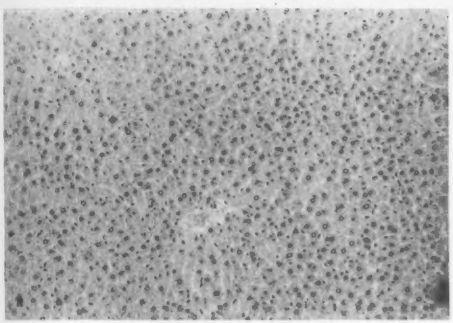


FIGURE 2.—*A* and *C*. Livers from non-tumor-bearing BDF_1 mice treated with NSC-122819. *B*. Normal liver.

Spleens were covered by a thickened fibrous capsule and occasionally showed lymphocytic depletion, resulting in decreased cytologic distinction between the malpighian corpuscles and the remaining splenic tissue. A bimodal splenic response was characterized by either splenic enlargement or a reduction in spleen size that paralleled decreases in liver weight. Decrease in kidney size ($P<0.01$) was occasionally observed also. Histologic alterations attributable to drug toxicity were not found in the kidney, intestine, lung, or bone marrow.

Thymic atrophy was noted, and frequently the organ was not grossly recognizable. Pancreatic atrophy, associated with substantial reductions in acinar cell cytoplasm and the virtual disappearance of zymogen granules, was observed (fig 4).

Animals with manifestations of delayed drug toxicity exhibited significant depression in serum levels of blood urea nitrogen (BUN), glucose, and total protein, as well as significant elevations in serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) (table 2).



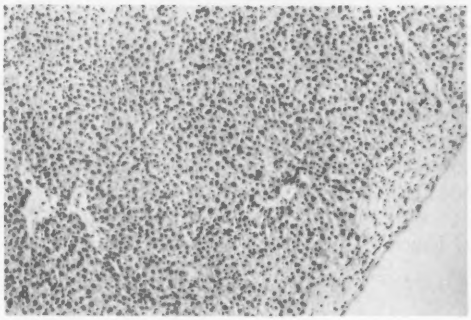


FIGURE 3.—*A*. Normal liver. *B*. Liver from a non-tumor-bearing BDF_1 mouse treated with NSC-122819 showing reduction in cytoplasmic volume and degenerative nuclear changes. *C*. Hepatic capsule from the animal treated with NSC-122819 showing mild round cell inflammation and moderate fibrosis. Hematoxylin and eosin stain, × 40.

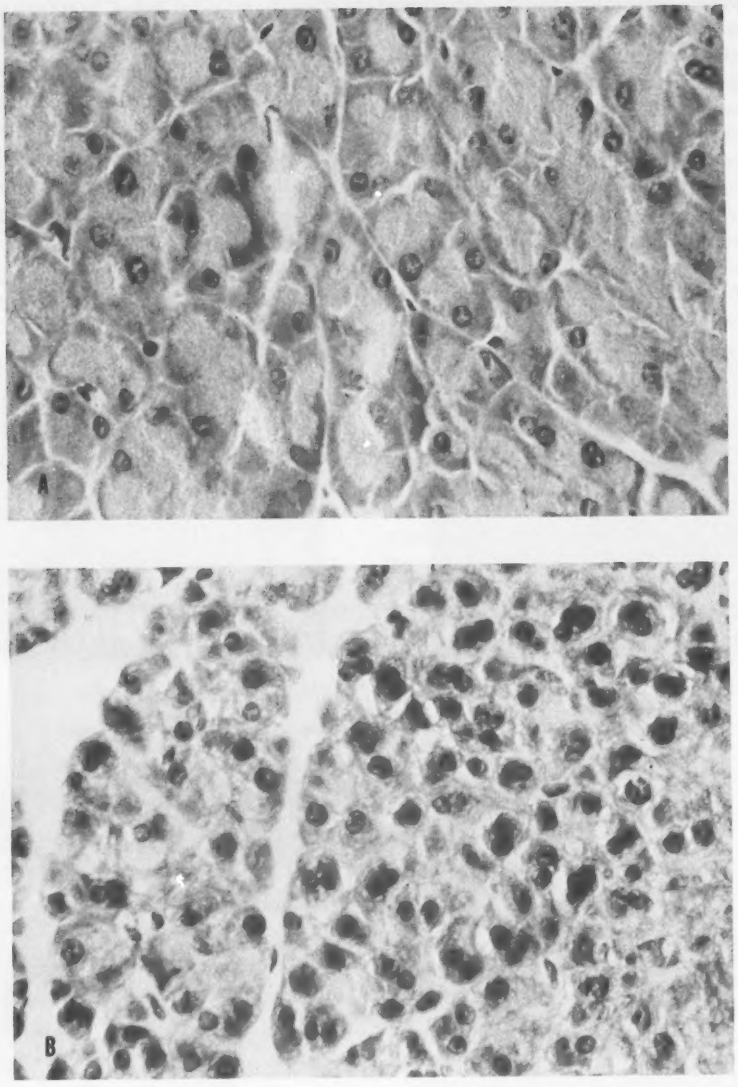


FIGURE 4.—*A*. Normal pancreatic acini with abundant cytoplasmic zymogen granules and peripheral basophilia. *B*. Pancreas from a non-tumor-bearing BDF_1 mouse treated with NSC-122819 showing loss of organized acinar pattern, absence of zymogen granules, and irregularly clumped cytoplasm. Hematoxylin and eosin stain, × 160.

TABLE 2.—Blood chemical values of BDF_1 mice treated with NSC-122819*

Treatment group	BUN (mg/100 ml)	Glucose (mg/100 ml)	Total protein (g/100 ml)	SGOT (units/100 ml)	SGPT (units/100 ml)
NSC-122819	35.9 ± 12.4‡	69.1 ± 49.4‡	3.4 ± 0.8‡	244 ± 157†	67 ± 23‡
(No. of animals)	(14)	(12)	(9)	(9)	(4)
Control	16.2 ± 2.7	168.5 ± 14.7	4.9 ± 0.1	93 ± 26	27 ± 13
(No. of animals)	(5)	(8)	(6)	(7)	(4)

*Mean ± 1 SD.
†$P < 0.025$.
‡$P < 0.005$.

Drug-Host Specificity

The BDF_1 mouse was used exclusively in the preceding studies. Since no NSC-122819-induced, delayed deaths had been reported previously, the possibility of drug-host specificity could not be ignored. Accordingly, NSC-122819 was administered to mice of six strains: A, C3H, CDF_1, DBA_2, C57BL, and BDF_1. Both early and delayed deaths were observed (table 3). Equally low incidences of early fatal toxicity were found in the CDF_1, DBA_2, and BDF_1 strains with one death among 20 treated mice. C3H mice had a 40% incidence of early deaths, which, by Chi-square analysis, exceeded that of the DBA_2, BDF_1, and CDF_1 strains ($P<0.01$), but was not statistically different from the incidence of early deaths in C57BL mice. The 65% incidence of early deaths for the A strain was significantly greater than the incidence for the C57BL strain ($P<0.005$).

Differences in survival time were also noted among the various strains (table 3). In order of decreasing survival time, mouse strains ranked as follows: C57BL, A, CDF_1, BDF_1, DBA_2, and C3H. Only mice of the C57BL strain survived treatment, and the inbred C3H mice proved to be the least resistant to NSC-122819.

TABLE 3.—Influence of mouse strain on length of survival following treatment with NSC-122819

Mouse strain	Dose (mg/kg)	No. of mice treated	Early toxic deaths		Late toxic deaths		Lifespan (days)	No. of survivors
			No. of deaths	Percent	No. of deaths	Percent†		
A	16	20	13	65	7	100	38.0 ± 9.6	0
	10	5	0	0	5	100	27.0 ± 3.9	0
C3H	16	20	8	40	12	100	24.9 ± 7.6	0
	10	5	0	0	5	100	36.7 ± 4.6	0
CDF_1*	16	20	1	5	19	100	33.4 ± 9.7	0
	10	5	0	0	5	100	37.2 ± 6.6	0
DBA_2	16	20	1	5	19	100	28.6 ± 6.8	0
	10	5	0	0	5	100	56.6 ± 9.1	0
C57BL	16	20	5	25	13	90	93.7 ± 40.0	2
	10	5	0	0	0	0		5
BDF_1†	16	20	1	5	19	100	31.8 ± 10.9	0
	10	5	0	0	5	100	81.8 ± 20.1	0

*The CDF_1 hybrid is a product of a C3H/HeJ × DBA_2/2J mating; the BDF_1 hybrid resulted from a C57BL/6J × DBA/2J cross.
†Percentage of survivors of early toxicity that succumbed to late toxicity as opposed to a percentage of the total number of treated mice.

Drug-Vehicle Interaction

The DMSO solvent did not induce early or delayed toxicity. To assist in determining whether deleterious drug-vehicle interaction might contribute to the toxicity, NSC-122819 in DMSO solvent was compared with preformulated NSC-122819. In the mouse strain studies just described, a dose of 16.0 mg/kg was administered in each solvent. Both early and late deaths resulted with each preparation. With respect to delayed deaths, mice treated with the DMSO preparation lived 40.1 ± 26.3 days on the average, and those treated with the preformulated preparation lived 39.8 ± 24.2 days. However, the incidence of early toxicity varied for the two preparations. A significantly greater incidence ($P<0.01$) of early death followed treatment with the preformulated preparation of NSC-122819. Thirty-five percent of the mice treated with this preparation died of acute toxicity, and at least one death occurred in each mouse strain. The DMSO preparation resulted in a 13% incidence of early deaths, and these deaths occurred only in the A and C3H strains.

Route of Drug Administration

Indications of chemical peritonitis following ip administration led to the testing of alternate routes of drug administration. Drug was dissolved in the DMSO-Tween-80 solvent and administered intramuscularly (im) into the shoulder or subcutaneously (sc) into the midcervical region. Nine of ten non-tumor-bearing mice remain alive and healthy 280 days after the start of treatment with NSC-122819 administered im at a dose of 16.0 mg/kg on Days 1, 4, and 7. One mouse died of tumor which developed in the area of drug administration. Ten animals that received the drug by the sc route remain alive and healthy.

Thirteen of 15 mice inoculated with 10^6 L5178Y leukemia cells are alive 142 days after sc administration of NSC-122819 at a dose of 10.0 mg/kg on Days 1, 4, and 7. One animal died on Day 17 and the other died on Day 42. These deaths were attributed to tumor growth. The mean survival time for 15 L1210-leukemia-bearing mice was 13.3 ± 0.7 days with a mean survival time of 8.0 ± 1.5 days for five untreated mice.

Although no evidence of either early or delayed toxicity was observed in these two studies, the sc administration of higher doses (18.0, 30.0, or 50.0 mg/kg) on the same schedule caused an acute, fatal toxicity in approximately 2 weeks for 40%–60% of non-tumor-bearing control mice. The mean survival time for L1210-leukemia-bearing mice treated with these and lower doses (18.0, 10.8, and 6.5 mg/kg) was 15 days or less.

DISCUSSION

In our laboratory, the oncolytic effectiveness of NSC-122819 for L1210 and L5178Y leukemia surpassed that of methotrexate, cytosine arabinoside, L-asparaginase, vincristine, and 6-mercaptopurine,[5] and several combinations of these agents given on similar treatment schedules. Within the dose range of 6.0–16.0 mg/kg, NSC-122819 completely eradicated tumor in 40%–90% of BDF_1 mice inoculated with 10^6 L1210 cells; at a dose of 2.2 mg/kg, the drug increased lifespan by 94%. Even greater oncolytic efficacy was demonstrated for L5178Y leukemia. The L5178Y tumor was eradicated in all mice treated with 3.6 mg/kg or more. At the lowest dose, 2.2 mg/kg, tumor was eradicated in 20% of the animals, and the mean increase in the lifespan of the succumbing mice was 171%.

In these studies, much of the drug's oncolytic effectiveness was offset by delayed toxicity which terminated in host death. The postmortem examination findings and abnormal blood chemical values of drug-intoxicated mice suggest that late toxic deaths may have resulted from a tissue-wasting process, but evidence

[5] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate; 6-MP.

for primary damage to a specific organ as the cause of death was not obtained. Small livers, spleens, and kidneys may have resulted from a general wasting of tissue, and the late thymic depletion and pancreatic changes may have been secondary to severe illness.

Although the pathologic lesion responsible for delayed toxicity has not been identified, evidence of chemical peritonitis has tentatively linked development of delayed toxicity to the ip route of drug administration. At doses of 3.6 mg/kg or lower, however, deaths from drug toxicity were not observed following ip administration and pronounced oncolytic activity was retained. Late toxic deaths were not observed following im or sc administration. With sc administration, tumor was eradicated in a high percentage of L5178Y hosts and the lives of mice inoculated with L1210 cells were extended by approximately 100%.

The pronounced oncolytic effectiveness of NSC-122819 against both L5178Y and L1210 leukemia appears to warrant continued investigation of this agent and other podophyllum analogs.

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Renal Clearance of Camptothecin (NSC-100880): Effect of Urine Volume [1,2]

Patrick J. Creaven and Larry M. Allen [3,4]

SUMMARY

The plasma levels of camptothecin after a single intravenous dose (67 mg/m² of body surface area) followed a biexponential decay. The theoretical minimum plasma levels at 24 hours after each dose of a daily × 5 course (C_{min}) were calculated from the experimentally determined slopes and intercepts of the plasma decay in seven patients. The experimentally determined C_{min} values deviated markedly from the theoretical pattern in five patients. A high urine volume increased the 24-hour urinary recovery of camptothecin and the uncorrected renal clearance in two patients studied and decreased the half-life of the excretion phase of plasma camptothecin from 15.31 to 7.00 hours in one patient. The variations in plasma levels and toxicity observed with camptothecin may be related to variations in renal clearance of the drug with fluctuations in urine volume.

[Cancer Chemother Rep 57:175–184, 1973]

Camptothecin (fig 1), an antineoplastic agent with a novel structure, has undergone phase I testing both here (1) and elsewhere (2), and has been evaluated in a phase II trial in carcinoma of the colon (3). We investigated the plasma levels of the drug in 15 patients who were treated on a daily × 5 schedule to determine if there were pharmacologic correlates for the reported variability in toxicity. The factor apparently most directly related to the variation in plasma levels and hematologic toxicity of the drug was the urine flow rate (4). We have therefore investigated the effect of urine volume on the urinary excretion and plasma levels of camptothecin.

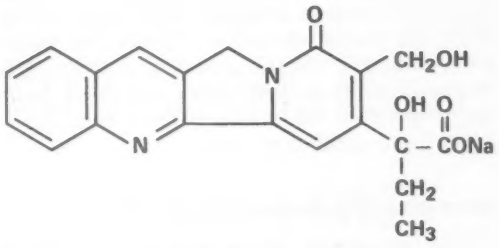


FIGURE 1.—Structure of camptothecin.

MATERIALS AND METHODS

Patient selection, drug source, and administration and determination of camptothecin are as previously described (4).

[1] Received Mar 3, 1972; revised Apr 16, 1973.

[2] Camptothecin: CAS reg. No. 25387-67-1. Supplied by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute (NCI).

[3] NCI-VA Medical Oncology Branch, Veterans' Administration Hospital, Washington, DC.

[4] The authors wish to thank Dr. C. Metzler of the Upjohn Company, Kalamazoo, Mich, for supplying his pharmacokinetic program NONLIN and for his help with making it operational, Mr. Meyer Gordon for many helpful discussions, and Mrs. G. Friedman for technical assistance.

Measurement of Uncorrected Renal Clearance

Renal clearance (Cl) was measured as described by Wagner (5), using the expression

$$Cl = \frac{Au_{t_2-t_1}}{\int_{t_1}^{t_2} C\,dt} \quad (1)$$

to calculate the uncorrected renal clearance, where $Au_{t_2-t_1}$ is the amount of drug excreted between time t_1 and t_2, and C is the plasma concentration of drug.

Plasma Levels After a Single Dose of Camptothecin

Plasma levels of camptothecin after a single dose (6.25–67 mg/m²) were fitted to the equation

$$C = Ae^{-\alpha t} + Be^{-\beta t} \quad (2)$$

where A and B are zero time intercepts and α and β are slopes of a semilogarithmic plot of the plasma decay by a nonlinear least squares regression program (NONLIN) on an IBM 360 digital computer. Theoretical minimum plasma levels (C_{min}) during a daily × 5 course in seven patients were calculated from the parameters obtained and from equation 2 using linear addition of terms.

Microsomal Metabolism of Camptothecin

The preparation of the microsomal fraction of rat liver and the study of the dealkylation of ethylmorphine and the hydroxylation of aniline are as previously described (6).

Camptothecin (10 μg/ml) was incubated with rat liver microsomes in the incubation mixture previously described (6). Aliquots were withdrawn from the assay mixture at intervals and assayed for *O*-hydroxylation (7), *N*-dealkylation (8), and camptothecin (4).

Aniline and ethylmorphine were incubated in the presence and absence of camptothecin and assayed for aniline *O*-hydroxylation and ethylmorphine *N*-demethylation respectively.

RESULTS

The decay of plasma camptothecin after a single intravenous dose fits the biexponential equation (equation 2). Figures 2 and 3 show the fit of observed and calculated plasma levels in two patients who received 67 mg/m², and figure 4 shows the data on the second of these patients when the dose was followed by intensive hydration. The observed C_{min} plasma levels of camptothecin previously reported (4) are shown graphically in figure 5*A*; the marked decrease in levels after fluid loading in three of the 15 patients (indicated by arrows) can be seen. The parameters of the plasma decay in seven of these patients are shown in table 1. Theoretical C_{min} levels of these patients who were treated daily × 5 are shown in figure 5*B* for comparison with the observed results. The excretion of camptothecin in 24 hours after treatment in two patients receiving normal and high fluid intake is shown in figures 6 and 7. Increasing the urine volume increased the percentage of camptothecin excreted in the first 24-hour period.

The relation between urine volume and uncorrected renal clearance of camptothecin in two patients is shown in table 2. A large increase in renal clearance was observed after increasing the urine flow rates. Studies using a rat liver microsomal system indicate that camptothecin does not affect the metabolism of either ethylmorphine or aniline (table 3).

DISCUSSION

The present study grew out of the observation that there was a large variation in the C_{min} levels and a suggestive relationship between these and the level of fluid intake in a series of patients receiving camptothecin on a daily × 5 schedule. Moreover, the C_{min} levels in a number of these patients did not follow the theoretically expected rise toward a plateau level (9) but showed marked deviations from it (fig 5*A*). The most striking deviations, moreover, were noted after hydration of three of these patients (as indicated by the arrows in fig 5*A*). It is possible to calculate the predicted C_{min} levels in patients receiving a drug with a long biological half-life if the equation for the decay and the parameters of the equa-

TABLE 1.—Parameters of plasma decay in 7 patients receiving camptothecin daily × 5

Patient No.	Dose (mg/m²)	Parameter	Parameter estimates: Estimate	SD	95% Confidence limits: Lower	Upper	Half-life (1) (hr)	Half-life (2) (hr)	Nonlinear least squares regression: ΣY²	ΣD²	Correlation	SD
1	6.25	A	1.438	0.168	0.972	1.905	1.07	11.58	11.11	0.017	0.995	0.065
		B	0.896	0.060	0.730	1.061						
		α	0.648	0.073	0.446	0.850						
		β	0.060	0.010	0.031	0.089						
4	11.2	A	1.771	0.083	1.411	2.131	0.99	39.26	11.69	0.001	0.999	0.027
		B	0.997	0.020	0.912	0.108						
		α	0.701	0.036	0.545	0.858						
		β	0.018	0.001	0.011	0.024						
5	15.0	A	1.560	0.305	0.775	2.342	0.66	12.59	87.92	0.112	0.995	0.150
		B	3.412	0.186	2.934	3.890						
		α	1.047	0.102	0.785	1.308						
		β	0.055	0.007	0.038	0.072						
6	15.0	A	1.614	0.052	1.481	1.747	1.66	8.99	9.89	0.004	0.999	0.027
		B	0.502	0.030	0.425	0.579						
		α	0.418	0.030	0.342	0.494						
		β	0.077	0.009	0.053	0.101						
13	20.0	A	4.097	0.326	3.380	4.814	1.16	25.64	65.25	0.398	0.992	0.190
		B	0.724	0.086	0.535	0.913						
		α	0.600	0.073	0.440	0.759						
		β	0.027	0.005	0.016	0.038						
14	20.0	A	4.974	0.286	4.274	5.674	1.39	13.74	204.6	0.153	0.998	0.160
		B	3.335	0.127	3.025	3.645						
		α	0.499	0.041	0.397	0.600						
		β	0.050	0.005	0.039	0.002						
15	20.0	A	5.753	0.575	4.472	7.033	2.21	10.74	349.4	1.188	0.993	0.345
		B	3.380	0.305	2.701	4.059						
		α	0.314	0.038	0.229	0.398						
		β	0.065	0.011	0.041	0.088						

TABLE 2.—Effect of urine flow rates on uncorrected renal clearance of camptothecin in 2 patients*

Patient No.	Urine flow rate (ml/hr)	Uncorrected renal clearance (ml/min)
1	49	12.24
	274	41.5
2	52	7.1
	114	43.7

*Uncorrected renal clearance was measured as described by Wagner (5) using equation 1. Camptothecin in urine was determined as described previously (4).

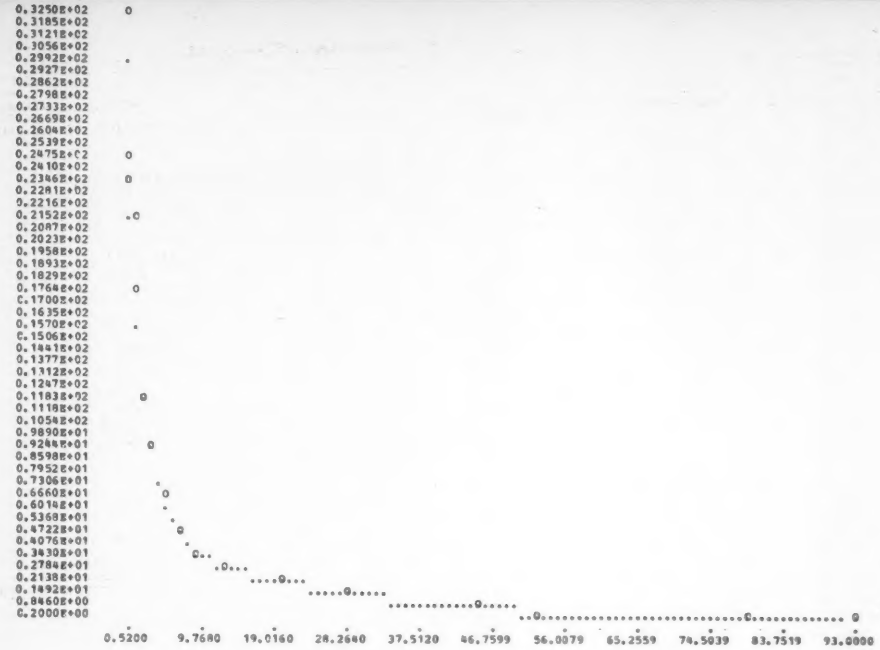
tion are shown. The decay of camptothecin is reported to be triexponential (2). However, when the plasma levels after a large single dose are studied for several days it is clear that the decay fits equation 2 (figs 2 and 3). We therefore determined the parameters of equation 2 for seven of these patients and calculated the predicted C_{min} values. These values correspond with the observed values in some cases but deviate markedly from it in others. The departure from the predicted values could be due to a number of causes. If camptothecin is metabolized, continued administration of the drug could theoretically induce the metabolism and cause a faster disappearance. The same effect would be produced by administration of phenobarbital, diphenylhydantoin, or other drugs known to induce microsomal metabolism. Although there was no evidence from previous studies of the existence of a metabolite of camptothecin (2,4), it is difficult to be certain that a drug whose reported recovery is less than 30% (2) is not metabolized when radioisotopically labeled drug is not available. We therefore examined the metabolism of camptothecin in an isolated microsomal enzyme system. No metabolism was seen and, after incubation, 100% of the drug was recovered unchanged from the incubate which was active in metabolizing other drugs. Drugs which are metabolized by the microsomal enzyme system bind to microsomal cytochrome. Since we lack the capability to investigate this directly, we studied it indirectly by examining the effect of camptothecin on the metabolism of ethylmorphine and aniline. If camptothecin binds to one of the active sites on microsomal cytochrome it will competetively inhibit the metabolism of either ethylmorphine, which undergoes type I binding, or of aniline, which undergoes type II binding. As can be seen from table 3, it does neither. Although these data do not prove conclusively that camptothecin is not metabolized, taken together with the failure of others (2) and ourselves (4) to demonstrate urinary metabolites of camptothecin, they are strongly suggestive that it is not metabolized, and they led us to investigate other possibilities.

Camptothecin is highly protein bound (4) and the concomitant administration of drugs which could displace it from binding sites on plasma protein might lead to more rapid excretion of free drug and, thus, decrease the biologi-

TABLE 3.—Effect of camptothecin on the dealkylation of ethylmorphine and the hydroxylation of aniline by rat liver microsomal fraction*

Substrate	Camptothecin	Rate of metabolism (μmol/g of liver/hr)
Aniline, 4.6 mM	0	0.26
Aniline, 4.6 mM	27μM	0.30
Ethylmorphine, 9 mM	0	1.98
Ethylmorphine, 9 mM	27μM	2.22

*Aniline was incubated with a rat liver microsomal mixture (6) for 15 minutes in air with shaking, and the formation of *p*-aminophenol was measured as described by Schenkman, Remmer, and Estabrook (7).



... ARE PREDICTED POINTS, O'S ARE OBSERVED POINTS

X	OBS. Y	CALC. Y	OBS-CALC	% DEVIATION	WEIGHT
0.52000E+00	0.32500E+02	0.30540E+02	0.19600E+01	6.03	0.10000E+01
0.80000E+00	0.25200E+02	0.27580E+02	-0.23700E+01	-9.40	0.10000E+01
0.11000E+01	0.24000E+02	0.24790E+02	-0.78000E+00	-3.25	0.10000E+01
0.16000E+01	0.22000E+02	0.20840E+02	0.11600E+01	5.27	0.10000E+01
0.21000E+01	0.17800E+02	0.17630E+02	0.17000E+00	0.96	0.10000E+01
0.32300E+01	0.12200E+02	0.12430E+02	-0.22000E+00	-1.80	0.10000E+01
0.41000E+01	0.94000E+01	0.97800E+01	-0.37000E+00	-3.94	0.10000E+01
0.55000E+01	0.71000E+01	0.70600E+01	0.40000E-01	0.56	0.10000E+01
0.75000E+01	0.51000E+01	0.50300E+01	0.70000E-01	1.37	0.10000E+01
0.96000E+01	0.40000E+01	0.40700E+01	-0.60000E-01	-1.50	0.10000E+01
0.12800E+02	0.32800E+01	0.32400E+01	0.40000E-01	1.22	0.10000E+01
0.20830E+02	0.25300E+01	0.23700E+01	0.16000E+00	6.32	0.10000E+01
0.29160E+02	0.16300E+01	0.17800E+01	-0.14000E+00	-8.59	0.10000E+01
0.45450E+02	0.10000E+01	0.10300E+01	-0.20000E-01	-2.00	0.10000E+01
0.53230E+02	0.67000E+00	0.79000E+00	-0.11000E+00	-16.42	0.10000E+01
0.79230E+02	0.28000E+00	0.33000E+00	-0.50000E-01	-14.29	0.10000E+01
0.93000E+02	0.20000E+00	0.20000E+00	0.0	0.0	0.10000E+01

SUM OF SQUARED OBSERVATIONS = 0.3419132E+04
SUM OF SQUARED DEVIATIONS = 0.1169771E+02
SUM OF WEIGHTED SQUARED DEVIATIONS = 0.1169771E+02
R-SQUARED = 0.997 COR= 0.997
S = 0.9485903 WITH 13 D.F.

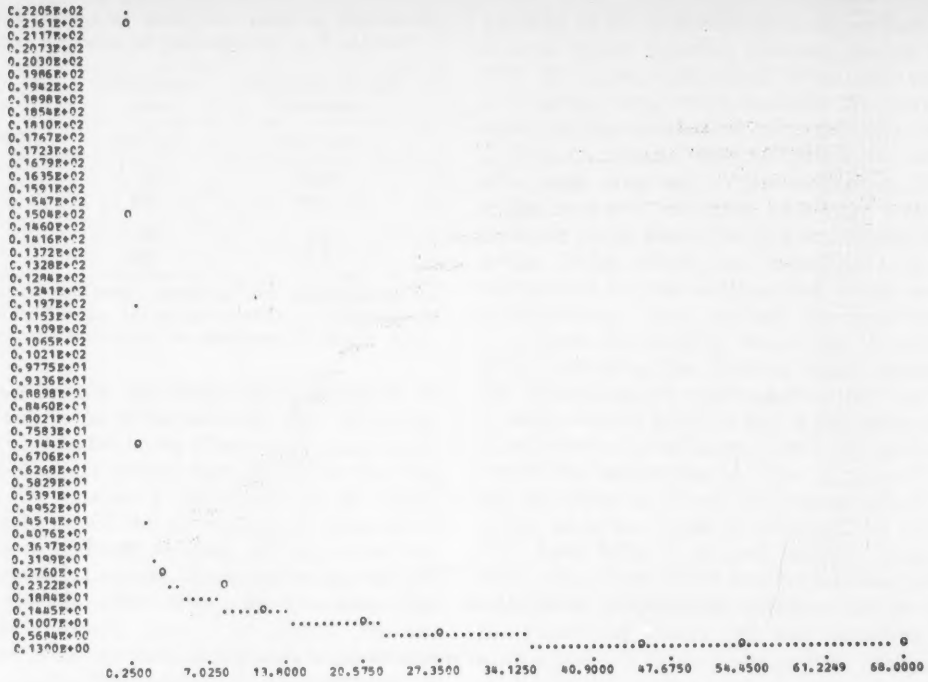
SUMMARY OF NON-LINEAR ESTIMATION

AFTER 4 ITERATIONS THE PARAMETER ESTIMATES THEIR STANDARD DEVIATIONS AND 95% CONFIDENCE LIMITS WERE:

NO.	ESTIMATE	STD. DEV.	95% CONFIDENCE LIMITS	
1	0.426245E+00	0.295262E-01	0.362458E+00	0.490032E+00 UNIVAR
			0.319944E+00	0.532546E+00 S PLANE
2	0.338732E-01	0.469292E-02	0.237347E-01	0.440116E-01 UNIVAR
			0.169776E-01	0.507687E-01 S PLANE
3	0.322462E+02	0.123116E+01	0.295821E+02	0.349103E+02 UNIVAR
			0.278066E+02	0.366859E+02 S PLANE
4	0.478377E+01	0.509277E+00	0.368355E+01	0.588400E+01 UNIVAR
			0.295026E+01	0.661729E+01 S PLANE

K1= 1.63 TK2= 20.46

FIGURE 2.—Predicted and observed plasma decay of camptothecin in 1 patient after 67 mg/m² was given iv. Observed plasma values (0) were fitted to equation 2 by a nonlinear least squares regression program as described in the Methods section. The parameters are $1 = \alpha$, $2 = \beta$, $3 = A$, and $4 = B$. K1 and TK2 are the half-lives of the initial and final decay phases respectively.



... ARE PREDICTED POINTS, O'S ARE OBSERVED POINTS

X	OBS. Y	CALC. Y	OBS-CALC	% DEVIATION	WEIGHT
0.25000E+00	0.21680E+02	0.22050E+02	-0.36000E+00	-1.66	0.10000E+01
0.75000E+00	0.15120E+02	0.14110E+02	0.10100E+01	6.68	0.10000E+01
0.15000E+01	0.72200E+01	0.78000E+01	-0.59000E+00	-8.19	0.10000E+01
0.30000E+01	0.29500E+01	0.35300E+01	-0.57000E+00	-19.32	0.10000E+01
0.90000E+01	0.23300E+01	0.18900E+01	0.45000E+00	19.31	0.10000E+01
0.12000E+02	0.16900E+01	0.16400E+01	0.40000E-01	2.38	0.10000E+01
0.21000E+02	0.13200E+01	0.10900E+01	0.23000E+00	17.42	0.10000E+01
0.28000E+02	0.80000E+00	0.79000E+00	0.10000E-01	1.25	0.10000E+01
0.45000E+02	0.35000E+00	0.37000E+00	-0.10000E-01	-2.86	0.10000E+01
0.54000E+02	0.24000E+00	0.24000E+00	0.0	0.0	0.10000E+01
0.68000E+02	0.15000E+00	0.13000E+00	0.20000E-01	13.33	0.10000E+01

SUM OF SQUARED OBSERVATIONS = 0.7700137E+03
SUM OF SQUARED DEVIATIONS = 0.2080296E+01
SUM OF WEIGHTED SQUARED DEVIATIONS = 0.2080296E+01
R-SQUARED = 0.997 COR= 0.998
S = 0.5451468 WITH 7 D.F.

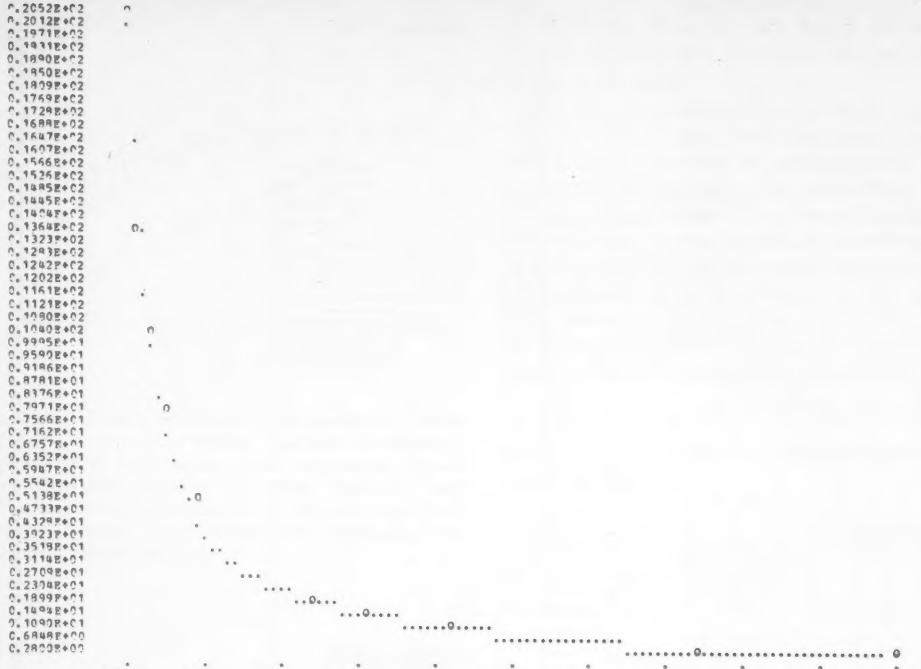
SUMMARY OF NON-LINEAR ESTIMATION

AFTER 10 ITERATIONS THE PARAMETER ESTIMATES THEIR STANDARD DEVIATIONS AND 95% CONFIDENCE LIMITS WERE:

NO.	ESTIMATE	STD. DEV.	95% CONFIDENCE LIMITS	
1	0.105256E+01	0.681844E-01	0.891345E+00	0.121381E+01 UNIVAR
			0.771208E+00	0.133395E+01 S PLANE
2	0.852618E-01	0.666523E-02	0.295009E-01	0.610226E-01 UNIVAR
			0.177571E-01	0.727664E-01 S PLANE
3	0.250553E+02	0.103833E+01	0.226000E+02	0.275106E+02 UNIVAR
			0.207705E+02	0.293401E+02 S PLANE
8	0.282172E+01	0.284618E+00	0.214870E+01	0.349474E+01 UNIVAR
			0.164722E+01	0.399623E+01 S PLANE

K1= 0.66 TK2= 15.31

FIGURE 3.—Predicted and observed plasma decay of camptothecin in a second patient after 67 mg/m² was given iv. *See* figure 2 for definitions of parameters.



... ARE PREDICTED POINTS, O'S ARE OBSERVED POINTS

X	OBS. Y	CALC. Y	OBS-CALC	% DEVIATION	WEIGHT
0.50000E+00	0.20530E+02	0.20210E+02	0.31000E+00	1.51	0.10000E+01
0.10000E+01	0.19730E+02	0.19137E+02	-0.57300E+00	-3.13	0.10000E+01
0.15000E+01	0.18580E+02	0.18530E+02	0.90000E-01	0.76	0.10000E+01
0.20000E+01	0.18020E+01	0.79100E+01	0.90000E-01	1.12	0.10000E+01
0.30000E+01	0.54900E+01	0.50300E+01	0.42000E+00	7.71	0.10000E+01
0.70000E+01	0.19200E+01	0.21600E+01	-0.23000E+00	-11.98	0.10000E+01
0.90000E+01	0.16000E+01	0.17300E+01	-0.12000E+00	-7.50	0.10000E+01
0.12000E+02	0.12300E+01	0.12800E+01	-0.50000E-01	-4.10	0.10000E+01
0.20500E+02	0.67000E+00	0.55000E+00	0.12000E+00	17.91	0.10000E+01
0.27500E+02	0.34000E+00	0.29000E+00	0.60000E-01	17.65	0.10000E+01

SUM OF SQUARED OBSERVATIONS = 0.8254922E+03
SUM OF SQUARED DEVIATIONS = 0.6997010E+00
SUM OF WEIGHTED SQUARED DEVIATIONS = 0.6997010E+00
R-SQUARED = 0.999 COR= 0.999
S = 0.3419990 WITH 5 D.F.

SUMMARY OF NON-LINEAR ESTIMATION

AFTER 2 ITERATIONS THE PARAMETER ESTIMATES THEIR STANDARD DEVIATIONS AND 95% CONFIDENCE LIMITS WERE:

NO.	ESTIMATE	STD. DEV.	95% CONFIDENCE LIMITS	
1	0.956424E+00	0.358155E-01	0.768987E+00	0.944262E+00 UNIVAR
			0.701459E+00	0.101179E+01 S PLANE
2	0.999773E-01	0.838365E-02	0.786610E-01	0.119493E+00 UNIVAR
			0.629297E-01	0.135225E+00 S PLANE
3	0.249719E+02	0.924051E+00	0.226104E+02	0.271330E+02 UNIVAR
			0.208484E+02	0.298752E+02 S PLANE
4	0.420356E+01	0.229388E+00	0.365455E+01	0.475262E+01 UNIVAR
			0.323183E+01	0.517569E+01 S PLANE

K1= 3.81 TK2= 7.00

FIGURE 4.—Predicted and observed plasma decay of camptothecin after 67 mg/m² was given iv during intensive hydration (same patient as in fig 3). *See* figure 2 for definitions of parameters.

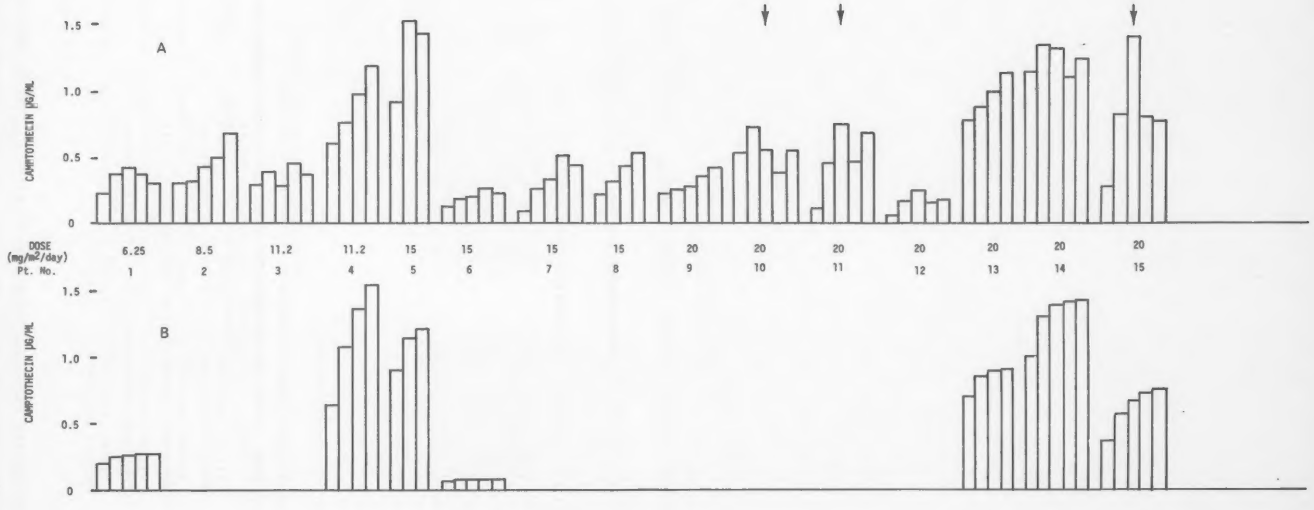


FIGURE 5.—Observed (*A*) and calculated (*B*) values of C_{min} during a daily × 5 course of camptothecin given iv in 15 patients. *A:* Experimentally determined C_{min} plasma levels during a daily × 5 course at the doses shown (4). *B:* Values derived from equation 2 and the parameters in table 1 by linear addition of terms. Arrows indicate points at which hydration of these patients was started.

% DOSE

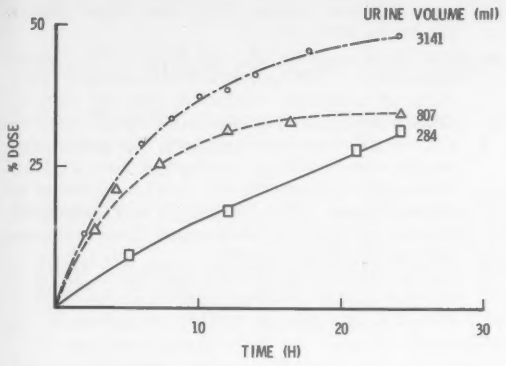


FIGURE 6.—Urinary recovery of camptothecin: effect of urine volume. The patient received camptothecin (67 mg/m² of body surface area) as a single iv dose on 3 separate occasions at 3-week intervals, and urine volumes were measured on low and high fluid intake. Urinary camptothecin was determined as previously described (4).

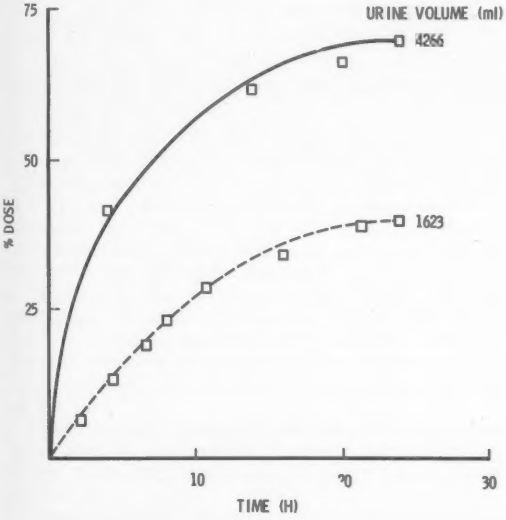


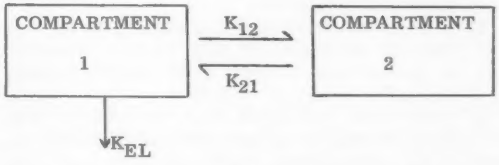
FIGURE 7.—Urinary recovery of camptothecin: effect of urine volume. The patient received camptothecin (67 mg/m² of body surface area) as a single iv dose on two separate occasions with a 3-week interval, and urine volumes were measured after moderate and high fluid intake. Urinary camptothecin was determined as previously described (4).

cal half-life. However, we found no evidence in our patients that this was the explanation of the effects seen.

The results presented in figures 6 and 7 and table 2 indicate that both the renal clearance and urinary recovery of camptothecin can be increased by increasing the urine flow. In one patient whose blood levels were studied after fluid loading, β was increased and the half-life of the second exponential decay phase decreased by fluid loading. With reference to this observation, however, it should be noted that β is a hybrid constant given in the two-compartment open model, which equation 2 describes, by

$$\beta = \tfrac{1}{2}(K_{12} + K_{21} + K_{EL} - \sqrt{(K_{12} + K_{21} + K_{EL})^2 - 4K_{21} \cdot K_{EL}})$$

where K_{12}, K_{21}, and K_{EL} are the transfer constants as shown:



The effect on β cannot, therefore, be taken as solely an effect on excretion, although it is probable from the other evidence presented that this is likely to be the major effect.

The studies presented here cannot be regarded as definitive because of the small number of patients studied, but they strongly suggest a marked effect of urine volume on camptothecin excretion. Such an effect is not unknown. It has been described for riboflavin (10) and is discussed by Fülgraff (11). However, it is perhaps not widely realized among chemotherapists that the renal excretion, plasma levels, and, therefore, not only the bladder toxicity but also the hematologic toxicity of a drug may be affected by fluid intake. We feel that this factor deserves further study and that urine volume should be more carefully controlled during initial clinical trials of new drugs if large variations in toxicity due to altered renal excretion are to be avoided.

Of interest in the present study is the finding that camptothecin decay is biexponential whereas the previous workers reported a triexponential decay (2). The reason for this dis-

crepancy is unclear. However, based on the results presented here it is possible to speculate that a change in the extent of hydration during the course of the study could be the cause. If β is increased by increasing urine flow rate, an initial intense hydration followed by a period of less intense hydration would lead to a change in β during the study and an apparent triexponential decay on a semilogarithmic plot. Although this is purely speculative, it does indicate the need to control factors such as urine volume if valid pharmacokinetic data are to be obtained.

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Phase I Study of ICRF-159 (NSC-129943) in Human Solid Tumors [1,2,3]

Robert E. Bellet,[4,5] Michael J. Mastrangelo,[4] Leon M. Dixon,[5] and John W. Yarbro [4,6,7]

SUMMARY

ICRF-159 was administered orally at a dose level of 0.25–1.25 g/m²/day in divided doses every 8 hours for 3 successive days to 19 patients with a variety of advanced solid tumors. Bone marrow suppression, manifested by significant leukopenia, was the dose-limiting factor. Thrombocytopenia was mild and transient; nausea and vomiting were well tolerated. Of 13 patients with measurable lesions, only one patient experienced tumor regression. When given orally at 8-hour intervals on 3 successive days, the maximum tolerated dose of ICRF-159 was 1.0 g/m²/day.

[Cancer Chemother Rep 57:185–189, 1973]

ICRF–159, a bisdiketopiperazine, is a nonpolar derivative of ethylenediaminetetraacetic acid (EDTA) (fig 1). This agent has demonstrated antitumor activity in murine L1210 leukemia [8] and has significantly inhibited spontaneous metastases in mice implanted with Lewis lung carcinoma (1). Although its mode of action has not been elucidated, inhibition of DNA synthesis (2) and alkylation [9] have been described. This paper details the results of a phase I study of ICRF–159 in adult solid malignant tumors.

MATERIALS AND METHODS

ICRF–159 was administered to 19 patients who had a variety of advanced solid malignant tumors. Histologic evidence of malignancy was required for entry into the study. Patients with a life expectancy of less than 3 months, prior major pelvic irradiation, or irradiation or systemic chemotherapy within 30 days of in-

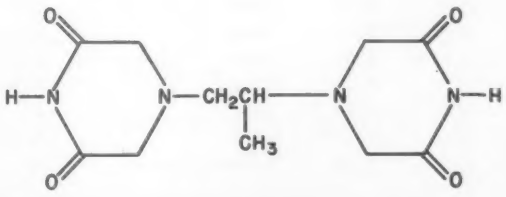

FIGURE 1.—Structural formula of ICRF-159.

[1] Received Sept 11, 1972; revised Apr 13, 1973.

[2] Supported in part by Public Health Service grants CA-05255 and CA-06551 (National Cancer Institute [NCI]) and RR-05539 (Division of Research Sources), National Institutes of Health, Department of Health, Education, and Welfare; and by an appropriation from the Commonwealth of Pennsylvania.

[3] ICRF-159 was supplied by the Cancer Therapy Evaluation Branch, Division of Cancer Treatment, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

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[8] Friedman MD, and Carter SK. Clinical brochure: ICRF-159, 1970. Prepared by the NCI, Bethesda, Md.

itiation of the study were ineligible for treatment. Patients with a hemoglobin level less than 9 g/100 ml, a white blood cell count (wbc) less than 4000 cells/mm³, a platelet count less than 100,000 platelets/mm³, a blood urea nitrogen (BUN) level greater than 25 mg/100 ml, a total serum bilirubin level greater than 1.5 mg/100 ml, or a serum glutamic oxaloacetic transaminase (SGOT) value greater than 120 units (normal = 5–40 units) were also ineligible.

The patients included 12 men and seven women. The mean age for men was 54 years (range, 43–87 years) and the mean age for women was 65 years (range, 50–77 years). Thirteen patients had received prior systemic chemotherapy.

The patients were treated with ICRF–159 given orally in divided doses every 8 hours for 3 successive days. Initially, two patients received a dose level of 0.25 g/m²/day which was 25% of the dose found to be "well tolerated and virtually non-toxic" in beagle dogs.[9] Drug dose levels were increased according to a modified Fibonacci scheme with two new patients receiving the drug at each higher dose level. No new patients were treated until toxicity at the previous dose level was clearly elucidated. Once the maximum tolerated dose range was determined, additional patients were studied to fully evaluate the spectrum of drug toxicity. Each patient was followed until all evidence of toxicity had disappeared; the minimum followup period was 4 weeks. Palpable lesions were measured weekly; pulmonary parenchymal tissue masses were evaluated with biweekly chest roentgenograms.

Hemoglobin determinations and wbc, platelet, and reticulocyte counts were obtained on Days 1 and 3 of therapy and then daily until values had returned to normal. Thereafter, weekly hemogram results were required. BUN, total serum bilirubin, SGOT, alkaline phosphatase, and serum calcium determinations were obtained before the study and thereafter at weekly intervals.

ICRF–159 was supplied in scored 250-mg tablets.

RESULTS

Of the 19 patients treated, 17 were evaluable for toxicity (table 1); two patients were

TABLE 1.—Summary of evaluable patients treated with ICRF-159

Patient No.	Sex, age	Tumor site	Type of prior therapy*	Prior chemotherapy	Dose level (g/m²/day) × 3 days	Total dose (g)
1	M, 64	Colon	S, C	5-FU, mitomycin C	0.25	1.25
2	M, 87	Lung	R	None	0.25	1.25
3	F, 69	Colon	S, C	5-FU	0.50	2.50
4	M, 51	Lung	R, C	Mechlorethamine	0.50	3.00
5	F, 60	Ovary	S, C	Melphalan	0.75	2.50
6	F, 77	Uterus	S, C	4-Drug combination†	0.75	3.75
7	M, 53	Colon	S, C	5-FU	0.75	3.75
8	M, 43	Lung	S	None	1.00	4.50
9	F, 75	Adenocarcinoma‡	C	Cyclophosphamide, 5-FU	1.00	5.00
10	M, 44	Lung	S, R	None	1.00	5.70
11	M, 51	Colon	S, C	5-FU	1.00	5.70
12	M, 44	Lung	C	CCNU, mechlorethamine	1.00	6.25
13	F, 56	Colon	S, R, C	5-FU	1.25	4.50
14	F, 50	Colon	S, R, C	5-FU	1.25	5.70
15	M, 49	Lung	C	Melphalan, procarbazine	1.25	6.25
16	M, 47	Colon	S, R	None	1.25	6.75
17	M, 60	Adenocarcinoma‡	R	None	1.25	7.50

*S = surgery, R = radiation therapy, C = systemic chemotherapy.
†Cyclophosphamide, vincristine, 5-FU, and methotrexate.
‡Primary unknown.

TABLE 2.—Toxic effects of ICRF-159*

Type of toxicity	Occurrence/total No. of patients
Hematologic	14/17
Leukopenia	14/17
Thrombocytopenia	3/17
Anemia	0/17
Gastrointestinal	8/17
Nausea	7/17
Vomiting	3/17
Diarrhea	2/17
Dermatitis	2/17
Alopecia	2/17
No. of deaths	0/17
No. of patients with no toxic effects	2/17
Total No. of patients with toxic effects	15/17

*Of 19 patients treated with ICRF-159, 17 were evaluable for toxicity.

unevaluable due to incomplete followup. The toxic effects experienced by the 17 evaluable patients are summarized in table 2. Two patients experienced no drug-related side effects; definite single or multiple drug-related toxic effects developed in 15 patients. No patient died during the study.

Hematologic toxic effects developed in 14 patients. All 14 patients experienced a dose-related decrease in the wbc to less than 50% of the pretreatment values (fig 2). The wbc nadir occurred on Day 12 (mean) with a range of 8–16 days. Wbc counts returned to normal 8 days (mean) thereafter with a range of 3–12 days. Seven patients experienced significant leukopenia of less than 2000 cells/mm³; however, in no patient did fever or signs of systemic infection develop. A greater than 50% decrease in platelet count occurred in three patients, which coincided temporally with the leukopenia. Thrombocytopenia was mild and transient; no platelet count was less than 55,000 platelets/mm³ and no hemorrhagic complications ensued. Anemia was not noted in any of the patients. A graphic representation of the hematologic toxicity experienced by a typical patient treated with ICRF–159 (1.25 g/m²/day for 3 days) is presented in figure 3.

Seven patients experienced drug-related episodes of nausea and three patients experienced vomiting. Nausea and vomiting were mild and self-limited; diarrhea developed in two patients. Two patients experienced significant alopecia. Cutaneous erythema and scaling over previously irradiated sites occurred in two patients. Pulmonary, renal, or hepatic toxicity was not noted and no patient experienced stomatitis or a significant decrease in serum calcium level.

Of 13 patients with measurable disease (five with bronchogenic carcinoma, four with aden-

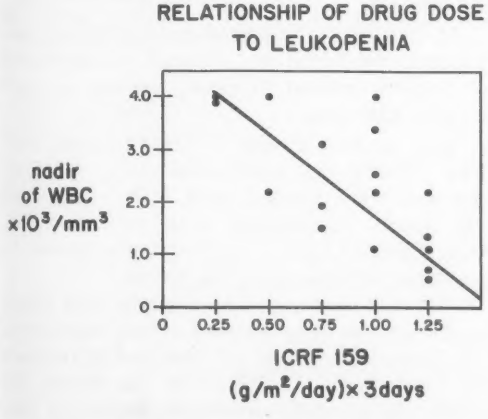

FIGURE 2.—Relationship of drug dose level to leukopenia.

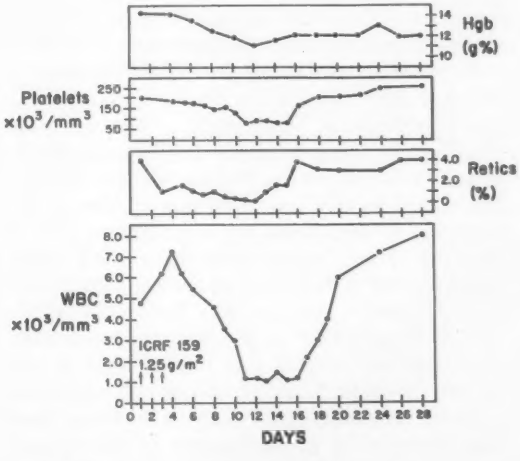

FIGURE 3.—Hematologic toxic reactions of patient No. 17.

ocarcinoma of the colon, two with metastatic adenocarcinoma of unknown origin, and one each with ovarian carcinoma and carcinosarcoma of the uterus), there was only one objective tumor regression. This objective remission occurred in patient No. 11, a 51-year-old man with adenocarcinoma of the colon metastatic to the lung. In spite of vigorous systemic chemotherapy with 5-fluorouracil (5–FU), pulmonary metastatic deposits increased in size. The patient was treated with ICRF–159 (1.0 g/m²/day) in divided doses every 8 hours for 3 successive days. The patient experienced mild nausea, vomiting, and diarrhea on Days 1, 2, and 3; the nadir of his leukopenia was 2600 cells/mm³ on Day 14. On Day 21, a chest roentgenogram revealed a greater than 50% decrease in the sum of the products of the two diameters of his four pulmonary nodules. Repeated courses of ICRF–159 were administered every 21 days and the patient remains in remission at 4 months.

DISCUSSION

Animal tumor data [8] revealed a maximum increase in lifespan of 128% against ascitic L1210 leukemia in mice when ICRF–159 was given every 3 hours, every 4 days, on Days 1, 5, and 9. In addition, Field et al (3) reported a 25-minute plasma half-life of ^{14}C-labeled drug in rats. Therefore, it was decided to administer ICRF–159 to humans in divided doses every 8 hours for 3 successive days (analogous to the every-3-hour-for-1-day schedule used in mice.)

Toxicologic studies of ICRF–159 in dogs [9] revealed anorexia, emesis, diarrhea, leukopenia, thrombocytopenia, moderate elevations in SGOT, and mild increases in BUN levels. Early clinical trials in Great Britain utilized ICRF–159 at a dose level of 20–30 mg/kg/day given orally in divided doses for a maximum of 3–4 days (4–7). A "sharp leukopenic effect" was noted as well as "thinning of the scalp hair"; no gastrointestinal toxicity was found. As a result of dose-related leukopenia, the maximum total dose per course was limited to 5 g. As shown in table 1, eight of our 17 evaluable patients received ICRF–159 in a total dose that exceeded 5 g; no patient in this group experienced life-threatening toxic effects when body surface area was used to determine the dose level.

A review of the combined British experience reveals that 61 patients with various hematologic and solid malignant tumors were treated with ICRF–159 administered at a multitude of dose levels and schedules and frequently in combination with methotrexate.[9] Leukopenia and thrombocytopenia occurred "within days" after beginning therapy. Other toxic effects included mucositis (6.5%), nausea and vomiting (5%), diarrhea (8%), and mild diffuse alopecia (6.5%). The present study demonstrated an 18% incidence of thrombocytopenia and an 82% incidence of leukopenia of early onset (Day 12) and of brief duration (8 days). Nausea and vomiting (41%) and diarrhea (12%) produced by this orally administered agent were not dose-limiting factors. Severe total alopecia (12%) was noted.

The appearance of cutaneous erythema and scaling over previously irradiated areas may be related to the findings reported by Creighton and Birnie (8) that in the mouse-embryo fibroblast system the behavior of ICRF–159 "strongly resembles that of radiomimetic drugs and ionizing radiation."

Since ICRF–159 is a derivative of the potent chelating agent EDTA, serum calcium level determinations were obtained frequently throughout the study. We did not find a significant decrease in the serum calcium level in any patient.

At dose levels that produced significant leukopenia, we found no evidence of stomatitis or toxicity related to renal, hepatic, or pulmonary functions.

Of 61 patients treated in Great Britain,[9] only nine patients had adult solid malignant tumors and were treated with ICRF–159 alone (ie, not in combination with methotrexate). One objective tumor remission was noted in a patient with cancer of the breast.

Although only one of 13 patients with measurable lesions experienced a tumor regression, our phase I study was not designed to evaluate antitumor activity. ICRF–159 has shown the ability to prevent neovascularization of primary tumors and thus to prevent metastases (1). It may well be a potent inhibitor of the

[8] *See* footnote 8.

tumor angiogenesis factor (TAF) (9). Therefore, the role of ICRF–159 in the treatment of cancer may lie in the area of adjuvant chemotherapy.

CONCLUSIONS

In this phase I study of orally administered ICRF–159, gastrointestinal and cutaneous toxic effects were mild and well tolerated. Bone marrow suppression, as manifested by significant leukopenia, was the dose-limiting factor. When given orally at 8-hour intervals for 3 successive days, the maximum tolerated dose of ICRF–159 was 1.0 g/m²/day. Since preclinical data [10] confirm that this G_2-M phase-active agent (2) is most effective when given intermittently, we plan to evaluate ICRF–159 in signal tumor groups by repeating 3-day courses at 21-day intervals.

LIST OF COMPOUNDS

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Ethylenediaminetetraacetic acid: NSC-2760; CAS reg. No. 6881-92-6; acetic acid, (ethylenedinitrilo)tetra-, disodium salt, dihydrate; EDTA; disodium edetate

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

ICRF-159: NSC-129943; CAS reg. No. 21416-87-5; 2,6-piperazinedione, 4,4′-propylenedi-, (±)-

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl, hydrochloride; HN2; nitrogen mustard

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride; Matulan

Vincristine: NSC-67574; CAS reg. No. 6449-03-2; leurocristine, sulfate (1:1)

[10] *See* footnote 8.

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Clinical Trials of *Cis*-diamminedichloroplatinum (NSC-119875)[1,2,3]

Alan J. Lippman, Christiane Helson, Lawrence Helson, and Irwin H. Krakoff[4]

SUMMARY

***Cis*-diamminedichloroplatinum was used in the treatment of 26 patients with advanced unresectable cancer who had failed to respond to conventional therapy. Various dose schedules were explored; dose levels were increased in graduated increments up to a total cumulative dose of 8.5 mg/kg. Toxic effects included myelosuppression, renal function impairment, high-frequency hearing loss, nausea, and vomiting. Seven patients did experience some therapeutic benefit.**

[Cancer Chemother Rep 57:191–200, 1973]

Cis-diamminedichloroplatinum is one of a group of transitional metal compounds which has shown antibiotic and antineoplastic effects in vitro (1,2) and in animal tumor systems (3–6). The mechanism of action appears to involve selective inhibition of DNA synthesis (7–10).

Studies in animals revealed toxic side effects that involved several organ systems including the bone marrow, the kidneys, and the gastrointestinal tract (11,12).[5] Early clinical experience also suggested ototoxicity (13).[6] At Memorial Hospital (New York, NY), *cis*-diamminedichloroplatinum was administered to 26 patients; the clinical data that was derived from that study furnished the basis for this report.

MATERIALS AND METHODS

Patient Selection

Twenty-six patients with a variety of malignant tumors received *cis*-diamminedichloroplatinum. All of the patients had histologically proven unresectable metastatic cancer and each patient had measurable areas of known cancer which served as objective criteria of response. Twenty-one patients completed an adequate course of therapy and are included in this report. An adequate course of therapy consisted of a sufficient amount of the drug to produce toxic side effects or a clinical response and a sufficient duration of followup to allow such effects to occur. Of the five patients who did not complete an adequate course of therapy, two voluntarily withdrew from the study and three died of their basic disease before sufficient time had elapsed for a clinical response or for side effects to occur.

The age and sex distribution, diagnosis, extent and duration of disease, and previous therapy of the patients are given in table 1.

[1] Received Nov 6, 1972; revised Feb 2, 1973.

[2] Supported by Public Health Service grants CA-05826 and CA-08748 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] *Cis*-diamminedichloroplatinum was supplied by the Cancer Therapy Evaluation Branch, Division of Cancer Treatment, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[4] Medical Oncology Service, Department of Medicine, Memorial Hospital for Cancer and Allied Diseases, and the Division of Chemotherapy Research, Sloan-Kettering Institute for Cancer Research, New York, NY.

[5] Broder L, and Carter S. Clinical brochure: *cis*-platinum (II) diamminedichloride, June 1971. Prepared by the DCT, NCI, Bethesda, Md.

[6] Hill J, Speer R, Loeb E, et al. Clinical experience with *cis*-platinous diamminedichloride. Presented at the VII Congress of Chemotherapy, Prague, Czechoslovakia, Aug 1971.

TABLE 1.—Summary of clinical data

Clinical data							Drug trial data			Toxic effects			
Patient No.	Age/ sex	Diagnosis/ region of disease	Extent of disease	Time from diagnosis to start of treatment (mos)	Survival after start of treatment (mos)	Previous treatment	Total dose (mg/kg)	Duration of treatment (days)	Response grade/ duration (mos)*	Hemato-poietic	Renal	Audio-logic	Nausea and vomiting
1	53/M	Epidermoid carcinoma/ nasopharynx	Local; nonresectable; cutaneous, lymph node, pulmonary metastases	60	5	Surgery, radiation, methotrexate, bleomycin	7.0	28	O–B/½	3	3	4	0
2	62/M	Adenocarcinoma/ colon	Skeletal, hepatic, pulmonary metastases	84	3	Surgery, radiation, 5–FU, thioTEPA	3.4	12	O–O	3	1	0	0
3	63/M	Epidermoid carcinoma/mouth	Local; cutaneous, subcutaneous metastases	9	2	Surgery, radiation, 5–FU, adriamycin, methasquin	3.3	24	O–O	1	2	0	3
4	42/M	Adenocarcinoma/ primary unknown	Hepatic; omental, mesenteric metastases	2	4	Surgery (laparotomy)	7.5	56	O–O	2	1	2	1
5	55/F	Epidermoid carcinoma/ tongue	Local; cutaneous, lymph node, pleural, pulmonary hepatic, skeletal, pancreatic, brain metastases	48	7	Surgery, radiation, bleomycin, methotrexate, cyclophosphamide, vincristine, methasquin	3.5	57	I–A/2	2	1	3	3
6	58/F	Melanoma	Pulmonary metastases	19	8	Surgery, BCNU + vincristine	5.5	17	O–O	3	2	0	1
7	32/F	(1) Hodgkin's disease	(1) Stage 4B	(1) 93	<1	(1) Radiation, vinblastine, chlorambucil, procarbazine	4.0	9	O–O	2	4	2	1
		(2) Acute myelomonocytic leukemia	(2) 40% blast cells	(2) 4		(2) Cytosine arabinoside, 6-thioguanine							
8	56/M	Adenocarcinoma/ prostate	Skeletal, pulmonary, hepatic metastases	60	5	Surgery, radiation, estrogen, aniline mustard, cyclophosphamide + prednisone, progesterone, mithramycin	4.0	8	O–A/2	3	3	1	1
9	26/M	Acute myelomonocytic leukemia	90% blast cells in marrow	38	7	Prednisone, cytosine arabinoside, 6-thioguanine, L-asparaginase, vincristine, methotrexate, BCNU, hydroxyurea, daunorubicin, adriamycin	7.0	23	O–O	1	1	1	1
10	71/M	Epidermoid carcinoma/tongue, larynx	Local; subcutaneous, mediastinal, pulmonary metastases	60	1	Surgery, radiation, cyclophosphamide	3.0	6	O–O	2	0	0	0
11	71/F	Epidermoid carcinoma/pharynx	Local; lymph node metastases	48	1	Surgery, radiation, bleomycin, methotrexate	2.5	5	O–O	3	2	Not evaluable	1
12	30/F	Embryonal rhabdomyosarcoma	Local; unresectable	5	3	Radiation, vincristine, cyclophosphamide + methotrexate	2.5	5	O–O	2	3	0	0

12	30/F	Embryonal rhabdomyosarcoma	Local; unresectable	5	3	Radiation, vincristine, cyclophosphamide + methotrexate	2.5	5	O–O	2	3	0	0
13	16/M	Acute lymphoblastic leukemia	90% blast cells in marrow	40	1	Prednisone, vincristine, methotrexate, 6-MP, cyclophosphamide, cytosine arabinoside, 6-thioguanine, adriamycin, L-asparaginase, 5-FU, daunorubicin, BCNU, vinblastine	4.0	8	O–O	2	2	0	0
14	54/F	Acute monoblastic leukemia	85% blast cells in marrow	12	<1	Cytosine arabinoside, 6-thioguanine, vincristine, BCNU, daunorubicin, methotrexate, adriamycin, L-asparaginase	3.5	7	O–O	4	4	Not evaluable	0
15	30/M	Epidermoid carcinoma/ nasopharynx	Local; unresectable; subcutaneous, lymph node metastases	60	‡	Radiation, cryosurgery, cyclophosphamide + vincristine + methotrexate, thioTEPA	8.0	109	O–C/1	3	3	0	2
16	37/M	Epidermoid carcinoma/ tonsil	Local; subcutaneous, lymph node, pulmonary, skeletal metastases	84	‡	Surgery, radiation, bleomycin, thioTEPA	6.0	150	II/6	3	0	0	1
17	52/F	Epidermoid carcinoma/ tonsil	Local; subcutaneous, thyroid lymph node, pulmonary metastases	38	7	Surgery, radiation, bleomycin, methotrexate, thioTEPA, 5-FU, prednisone	5.0	59	O–C/½	2	2	2	2
18	53/M	Epidermoid carcinoma/ nasopharynx	Local; unresectable	27	‡	Surgery, radiation, bleomycin + methotrexate, VP-16	2.0	2	O–O	2	2	0	2
19	42/F	Epidermoid carcinoma/ mouth	Local; unresectable, subcutaneous, lymph node metastases	10	2	Surgery, radiation	3.0	26	O–O	2	0	0	0
20	68/M	Epidermoid carcinoma/ mouth	Lymph node; pulmonary metastases	4	1	Surgery, radiation	2.0	4	I–A	1	2	0	0
21	62/M	Leiomyosarcoma	Local; recurrent, subcutaneous, hepatic, pulmonary, skeletal metastases	96	2	Surgery, radiation, cyclophosphamide, adriamycin, 5-azacytidine	5.0	40	O–O	1	2	0	1

*See ref 14.
†See appendix for definitions.
‡Living at time of this report.

All of the patients had previously recovered from the toxic side effects of any prior therapy before *cis*-diamminedichloroplatinum was administered.

Twelve patients received a single course of therapy; five received two separate courses; three received three courses; and one received four courses. Thus, a total of 35 individual courses of therapy were evaluable for toxicologic study.

Methods

All patients were treated in the hospital under close observation. Daily total fluid intake and output were recorded and parenteral supplementation was given, if necessary, to preclude clinical dehydration. The following parameters were followed: daily urinalysis, leukocyte and platelet counts, creatinine, blood urea nitrogen (BUN) and uric acid levels; semiweekly examinations of serum calcium, phosphorus, electrolyte, total protein, albumin, serum glutamic oxaloacetic transaminase (SGOT), lactic dehydrogenase (LDH), alkaline phosphatase, and 5′-nucleotidase levels. Appropriate studies were obtained on all patients who showed an indication of renal function impairment based upon a rise in BUN and creatinine values.

Audiograms were obtained on all of the patients prior to therapy and thereafter at weekly or biweekly intervals. Threshold hearing was serially determined at 250–8000 cps with the use of a portable Beltone model 12D audiometer, calibrated to 1964 ISO values. Because of their poor physical conditions, all of the patients were examined in their hospital beds or rooms. Background noise interfered with accurate bone conduction readings, which were not pursued serially. The individual hearing thresholds were determined with descending intensities on alternate ears for each frequency. The frequencies were tested in the following sequence: 4000, 1000, 8000, 500, 250, 2000, 8000, and again at 1000 cps as an internal control.

Prior to the start of therapy, renal function was normal in all of the patients according to intravenous urogram results, urinalysis reports, and creatinine clearance determinations.

The scheduled dose of *cis*-diamminedichloroplatinum was dissolved in physiologic saline at a concentration of 1 mg/ml and was administered by intravenous (iv) infusion over a 5–10-minute period. In the first two patients treated, the initial dose was calculated to represent one tenth of the minimal toxic dose in mice (0.03 mg/kg/day) [†] and was increased by graduated increments at 4-day intervals until toxic side effects occurred. Subsequent patients received various daily dose levels between 0.25 and 2.0 mg/kg for total course doses of 1.25–7.0 mg/kg.

One patient was treated concurrently with bleomycin. No other patient received concurrent chemotherapy or radiation therapy.

Treatment was discontinued in any patient who failed to show a therapeutic response after toxic side effects had occurred. Nine patients received more than one course of therapy. Five of these patients initially showed no definite toxic effects. Four patients who experienced therapeutic responses after the first course of therapy received one or more additional courses when recovery from toxic side effects was apparent and their disease showed objective progression. All patients eventually manifested some form of toxicity.

Even if a therapeutic benefit was obtained, further administration of *cis*-diamminedichloroplatinum was suspended in any patient who showed incomplete recovery of renal function, myelosuppression, hearing loss, or intolerable nausea and vomiting.

RESULTS

Toxic Effects

Clinically significant toxic side effects included myelosuppression, renal function impairment, high-frequency hearing loss, nausea, and vomiting. One patient (No. 1) who received a total dose of 7.0 mg/kg in a single 28-day course showed transient mild elevations of SGOT and LDH levels (with recovery to baseline values within 5 days) and partial alopecia. These effects were not noted in any other patient.

Stomatitis, dermatitis, cystitis, and diarrhea were not observed. Cardiac, pulmonary, cen-

[†] *See* footnote 5.

tral nervous system, or allergic reactions did not occur.

Hematologic Observations

The patterns of the hematologic toxic effects are given in table 2. For purposes of analysis, the 35 individual courses of therapy were retrospectively divided into two groups (high and low total dose per course). Sixteen patients received a total course dose of 3.0–7.0 mg/kg and constituted the high-dose group; 19 patients received a total course dose of 1.25–2.5 mg/kg and constituted the low-dose group.

Leukopenia and thrombocytopenia occurred in all but six patients in the 35 individual courses. Four of these patients were in the low-dose group. In the other 29 courses, varying degrees of myelosuppression were noted.

In general, the higher doses produced a more severe myelosuppression but this finding was not consistently predictable. The lowest total dose which produced myelosuppression was 1.5 mg/kg given in divided doses over a period of 6 days, although in one patient 4.0 mg/kg given in 8 days did not cause definite myelosuppression.

Leukopenia with a nadir of less than 2000 cells/mm^3 developed in six of 16 patients who had received more than a 3.0-mg/kg course, whereas only three of 19 patients who had received less than a 3.0-mg/kg course manifested that degree of leukopenia. Leukopenia did not develop in four of the 16 patients who received more than a 3.0-mg/kg course; however, eight of 19 patients who received less than that amount showed no change in the white blood cell count (wbc). The findings were similar for platelet counts. Thrombocytopenia developed in six of 16 patients in the high-dose group with platelet counts of less than 50,000 cells/mm^3. In contrast, only one of 19 patients in the low-dose group had thrombocytopenia of this magnitude.

Maximal myelosuppression occurred on an average of 23 days (range 10–39) after the inception of therapy. All patients had fully recovered by approximately 39 elapsed days (range 13–62).

The onset of myelosuppressive activity, the nadir of leukopenia or thrombocytopenia, the degree of myelosuppression, and the time to full recovery did not vary significantly with the total dose given per course or the duration of therapy for the individual course.

In patients who received more than one course, subsequent courses did not produce a more profound myelosuppression nor did they result in a more prolonged recovery. Two patients received a subsequent course prior to full hematologic recovery from an earlier course, and, although both recovered fully, each experienced a slightly more severe and prolonged myelosuppression.

In four patients with leukemia, two showed a decrease in wbc. Patient No. 7 initially had a wbc of 19,700 cells/mm^3. After a total of 4.0 mg/kg given in 9 days the wbc decreased to a nadir of 3800 cells/mm^3. Patient No. 14 initially had a wbc of 62,000 cells/mm^3. After a total of 3.5 mg/kg in 7 days the wbc decreased to a nadir of 1800 cells/mm^3. Two other patients with leukemia (patients No. 9 and No. 13) showed no change in wbc.

In four patients with acute leukemia who were thrombocytopenic as a basic feature of their disease, supportive platelet transfusions were given during the *cis*-diamminedichloroplatinum therapy. No other patients required supportive platelet transfusions. Packed red blood cell transfusions were given in only a few instances where intercurrent, incidental, symptomatic anemia developed. Hemolytic anemia was not noted in any of the patients.

Severe leukopenia (less than 2000 cells/mm^3) developed in one patient (No. 14) who died of intercurrent infectious complications on Day 15 when her leukocyte count was rising. Drug-induced leukopenia was felt to have contributed to her death.

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TABLE 2.—Characteristics of hematologic toxicity

No. of courses	Total dose (mg/kg)	Schedule (mg/kg/day)	Duration of treatment (days)	No. of patients with leukopenia; nadir				Mean day of leukocyte nadir (range)	Mean day of leukocyte recovery (range)	No. of patients with thrombocytopenia; nadir				Mean day of platelet nadir (range)	Mean day of platelet recovery (range)
				<2000 cells/mm³	2000–4000 cells/mm³	>4000 cells/mm³	No leukopenia			<50,000 cells/mm³	50,000–150,000 cells/mm³	>150,000 cells/mm³	No thrombocytopenia		
16	3.0–7.0	0.25–1.0	2–28	6	3	3	4	23.8 (10–45)	39.2 (16–54)	6	2	3	5*	23.2 (14–39)	42.3 (19–56)
19	1.25–2.5	0.25–2.0	1–6	3	6	2	8	23.2 (14–39)	37.2 (20–62)	1	6	8	4	22.9 (10–34)	37.0 (13–62)

*Includes 3 patients with thrombocytopenia related to acute leukemia who received supportive platelet transfusions.

with *cis*-diamminedichloroplatinum and continued to rise until the time of death. Both of these patients, however, suffered from acute leukemia, had generalized sepsis and metabolic imbalances, and were being treated concurrently with known nephrotoxic antibiotics (gentamicin and amphotericin B). Nevertheless, therapy with *cis*-diamminedichloroplatinum was considered at least contributory to their renal failure. No other patients received a known nephrotoxic drug concurrently with *cis*-diamminedichloroplatinum.

In the low-dose group, ten of 19 patients showed no change in BUN or creatinine levels. Two patients showed persistent elevations of each, and all other patients showed only transient elevations with complete recovery to baseline values.

The peak of the rise in BUN and creatinine values was seen in the high-dose group on Day 13 after the inception of therapy. Recovery to baseline values was observed in most patients by Day 27. In addition to the two deaths mentioned earlier, four other patients (Nos. 1, 8,11, and 15) showed persistent elevations above the baseline values of BUN and creatinine. Two of these patients (No. 8 and No. 11) received single courses at dose levels of 4.0 and 2.5 mg/kg, respectively. One patient (No. 1) received the highest total dose per course, 7.0 mg/kg, in gradually increased increments over a 28-day period. One patient (No. 15) received three separate courses during which his creatinine clearance gradually declined from 126 to 80 ml/minute.

In the low-dose group, peak BUN and creatinine values were observed around Day 8 and full recovery occurred around Day 21 in all but one patient. That patient (No. 15) had received more than one course of treatment and appeared to manifest cumulative deterioration in renal function.

In the two patients who died in a state of uremia (No. 7 and No. 14), histopathologic examination of the kidneys revealed tubular necrosis in each patient. Seven other patients who died were also subjected to postmortem examination. Renal histopathologic examination by light microscopy revealed a variety of lesions explainable by mechanisms related or unrelated to their basic disease; however, renal tubular necrosis was seen in only one patient (No. 5). That patient had received four separate courses of therapy for a total cumulative dose of 8.5 mg/kg over a 57-day period. Although only a transient rise in the BUN level occurred after the fourth course without a corresponding rise in creatinine, histopathologic examination of the kidneys disclosed focal regeneration of tubular epithelium.

No significant differences in toxicity were found when the treatment schedules were varied. Only one patient (No. 15), of those who received more than one course of therapy, manifested evidence of cumulative toxicity with a decrease in creatinine clearance.

Audiologic Data

Apparently irreversible high-frequency, bilateral hearing loss (which we presumed to be neural) occurred in eight patients. In 11 patients, no hearing loss was discerned and in two patients, audiometric data could not be satisfactorily obtained because of the patients' inability to cooperate.

One patient (No. 1) experienced clinically apparent hearing loss with a 40–80-decibel loss in the speech range (1000–4000 cps). This patient was the first treated and had received daily injections in increments up to 1.0 mg/kg on 28 consecutive days before his loss of hearing became apparent. No other patient experienced a hearing loss in the speech range; thus, hearing loss was detected solely on serial audiograms and only in the 4000–8000-cps range.

The ototoxicity data are summarized in table 4. Hearing loss occurred only at cumulative total doses which exceeded 4.0 mg/kg. In patients who received more than one course of therapy, hearing loss appeared to be additive and became more severe with each course. On no occasion did audiograms clearly improve once the hearing loss became established. One patient, who received a total dose of 4.0 mg/kg in 8 days, survived 3 months after his last dose of *cis*-diamminedichloroplatinum. His final audiogram, taken 2 weeks prior to his death, suggested a slight improvement in the high-frequency range.

It was observed that unilateral hearing losses

TABLE 3.—Characteristics

No. of courses	Total dose (mg/kg)	Schedule (mg/kg/day)	Duration of treatment (days)	Degree of toxicity* (No. of patients) 4+	3+	2+	1+	0	Mean baseline BUN (mg/100 ml) (range)
16	3.0–7.0	0.25–1.0	2–28	2	2	10	2	0	14.2 (7–28)
19	1.25–2.5	0.25–2.0	1–6	0	2	4	3	10	12.2 (6–21)

*See appendix for definitions.

TABLE 4.—Summary of ototoxicity data*

Total cumulative dose (mg/kg)	No. of patients	Degree of ototoxicity
2.0–3.9	4	None observed
4.0–5.9	6	0, 0, 1, 2, 2, 3
6.0–7.9	4	0, 1, 2, 4
8.0–8.5	2	0, 3

*See appendix for definitions.

occurred in two patients. These losses appeared to be conductive and related to intercurrent otitis. Hearing loss in both of these patients was reversible after appropriate therapy.

Gastrointestinal Reactions

Mild nausea and vomiting, which occurred 2–48 hours after the drug was administered, was seen in 22 courses of treatment and was easily controlled with mild antiemetics. On three occasions, vomiting was moderate and required parenteral alimentation for 24–48 hours. In two patients severe vomiting required the discontinuation of therapy. Both patients had epidermoid carcinomas of the mouth with impairment of swallowing, and therapy was discontinued due to fear of aspiration. Seven patients, including one who received two courses of therapy, experienced no gastrointestinal side effects.

Therapeutic Response

Seven patients (33%) experienced some therapeutic benefit, as indicated in table 1. Responses were graded according to the standard criteria of Karnofsky (14).

All except one of the responses occurred in patients with epidermoid carcinomas of the head and neck region, and all responses were of brief duration. Two patients showed a I–A response (patients No. 5 and No. 20). Patient No. 5 showed further progression of disease after an initial response of 8-weeks' duration; patient No. 20 showed a response at 3 weeks but died of intercurrent pneumonia during the 5th post-therapy week. One patient showed stabilization of his disease for 6 months after which his disease progressed. Three patients showed objective responses of less than 4-weeks' duration.

One patient with adenocarcinoma of the prostate experienced subjective partial relief of skeletal pain for 8 weeks but did not manifest any objective improvement.

No other therapeutically useful responses in the variety of solid tumors and leukemias were observed. One patient (No. 19) received *cis*-diamminedichloroplatinum and bleomycin concurrently but experienced only hematologic toxic effects; no therapeutic benefit was derived.

CONCLUSIONS

Early clinical trials with *cis*-diamminedichloroplatinum have demonstrated significant toxic side effects and modest therapeutic benefits.

Limiting toxic effects appear to be cumulative renal function impairment and apparently irreversible, high-frequency hearing loss. Myelosuppression appears to be reversible. Toxicity is only roughly dose-related. No idiosyncratic reactions were observed.

Of 21 evaluable patients, seven experienced minor therapeutic benefits. It is of interest that six of these responses were observed in patients with epidermoid carcinomas of the

Mean baseline creatinine (mg/100 ml) (range)	Mean peak BUN (mg/100 ml) (range)	Mean peak creatinine (mg/100 ml) (range)	Mean day of peak BUN (range)	Mean day of peak creatinine (range)	Mean recovery of BUN (mg/100 ml) (range)	Mean recovery of creatinine (mg/100 ml) (range)	Mean day of recovery of BUN (range)	Mean day of recovery of creatinine (range)
0.9	38.5	2.2	12.8	14.5	17.5	1.2	27.0	26.3
(0.7–1.4)	(13–113)	(1.2–5.0)	(5–31)	(4–58)	(8–40)	(0.6–2.5)	(9–107)	(10–107)
0.8	30.1	1.7	8.3	8.0	15.8	1.0	21.3	17.0
(0.6–1.0)	(17–56)	(1.3–2.9)	(5–14)	(5–13)	(6–29)	(0.6–1.4)	(8–40)	(8–33)

head and neck. Although the responses were limited in degree and duration, they demonstrated the definite antitumor potential of *cis*-diamminedichloroplatinum. Further studies of this drug in combination with other agents are being undertaken.

APPENDIX.—Definitions of toxicity

Hematologic toxicity

4+	Wbc < 2000 cells/mm³, platelet count < 50,000 platelets/mm³; patient died as a proximal result of myelosuppression
3+	Wbc < 2000 cells/mm³, platelet count < 50,000 platelets/mm³; patient recovered
2+	Wbc 2000–4000 cells/mm³, platelet count 50,000–150,000 platelets/mm³
1+	Wbc > 4000 cells/mm³, platelet count > 150,000 platelets/mm³
0	No change in blood cell counts

Renal toxicity

4+	Fatal
3+	BUN level rose to more than twice the baseline level and remained elevated; creatinine rose above baseline level and remained elevated
2+	BUN level and creatinine rose above baseline levels but later returned to baseline
1+	Slight transient rise in BUN level; no rise in creatinine
0	No change in BUN level or creatinine

Audiologic toxicity

4+	Clinically apparent 40–80-decibel loss in speech range (1000–4000 cps)
3+	Clinically inapparent 40–80-decibel loss in 4000–8000-cps range
2+	20–40-decibel loss in 4000–8000-cps range
1+	10–20-decibel loss in 4000–8000-cps range
0	No change

Gastrointestinal toxicity

4+	Fatal
3+	Severe; required interruption of therapy
2+	Moderate; required parenteral alimentation
1+	Mild; easily controlled with antiemetics
0	None

LIST OF COMPOUNDS

Adriamycin: NSC-123127

Aniline mustard: NSC-18429; CAS reg. No. 553-27-5; aniline, *N,N*-bis(2-chloroethyl)-

L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3

5-Azacytidine: NSC-102816; CAS reg. No. 320-67-2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Bleomycin: NSC-125066

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-

***Cis*-diamminedichloroplatinum:** NSC-119875; CAS reg. No. 1853-33-9

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; daunomycin (NSC-82151)

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate

Methasquin: NSC-122870; aspartic acid, *N*-[*p*-[[(2,4-diamino-5-methyl-6-quinazolinyl)methyl]amino]benzoyl]-, hydrate (5:3), L-

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Mithramycin: NSC-24559; CAS reg. No. 18378-89-7

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

VP-16: NSC-141540; epipodophyllotoxin, 4′-demethyl-, 9-(4,6-*O*-ethylidene-β-D-glucopyranoside)

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Treatment of Advanced Neoplastic Disease With 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU; NSC-79037) [1,2,3]

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SUMMARY

CCNU was administered orally at a dose level of 130 mg/m² to 59 patients with advanced neoplasms. Fifty-three of the 59 patients were considered evaluable. Two of seven patients with Hodgkin's disease which was resistant to combination chemotherapy achieved complete remissions which lasted 9+ and 13+ months respectively, while two other patients had brief periods of subjective improvement. One of 12 patients with carcinoma of the colon had a dramatic objective improvement which lasted 9 months. Objective partial responses which ranged from 2 to 6 months in duration were seen in patients with non-Hodgkin's lymphoma, brain tumors, ovarian teratoma, malignant melanoma, plasma cell leukemia, and cancer of the cervix. Subjective responses were observed in six other patients. Transient nausea and vomiting occurred frequently after the drug was administered. Renal or hepatic toxicity was not observed. The major toxic effect was delayed hematologic suppression similar to that observed with BCNU. Nadir granulocyte levels (less than 750 cells/mm³) occurred in 13% of the patients and thrombocytopenia (less than 50,000 platelets/mm³) in 23% 24–26 days after treatment. Prior chemotherapy increased the incidence of severe toxic effects. One drug-related death resulted from infection in a patient who was severely leukopenic and who had received intensive prior chemotherapy. Cumulative hematologic depression did not occur when CCNU was administered at this dose level at 6–8-week intervals.

[Cancer Chemother Rep 57:201–207, 1973]

The nitrosoureas are a unique group of antineoplastic agents with significant activity in a wide spectrum of animal and human neoplasms. Interest in these agents was generated by the discovery that the nitrosoureas were effective in the treatment of mice which

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[2] Supported in part by Public Health Service grants CA-8341 and CA-5138 from the National Cancer Institute (NCI), National Institutes of Health (NIH), Department of Health, Education, and Welfare.

[3] CCNU was supplied by the Cancer Therapy Evaluation Branch, Division of Cancer Treatment, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

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[5] We are indebted to all the members of the Section of Oncology and Cancer Chemotherapy who participated in the care of these patients and to Drs. Ernest Kohorn, David S. Fischer, Leonard Farber, Rose J. Papac, Harold W. Schell, Jr., and Norman L. Cressey for permission to study their patients.

had been implanted with intracerebral as well as intraperitoneal L1210 leukemia (1,2). Lipid solubility and penetration of the blood-brain barrier were demonstrated (3). Preclinical trials showed the nitrosourea BCNU to be effective in a number of central nervous system tumors, in a variety of sarcomas and ascitic carcinomas, and in certain tumors which had acquired resistance to cyclophosphamide (4). Studies with BCNU in man have shown antineoplastic effects in lymphomas, malignant melanoma, Ewing's sarcoma, acute and chronic leukemia, primary and metastatic brain tumors, and carcinomas of the head and neck, gastrointestinal tract, breast, and lung (5). Patients with advanced Hodgkin's disease resistant to therapy with conventional alkylating agents achieved clinically useful responses (6–8).

These data have led investigators to synthesize other agents derived from the original compound, 1-methyl-1-nitrosourea. One of these analogs, CCNU, has demonstrated superior activity in various animal tumor systems. Alteration of the structure of BCNU by replacement of a chloroethyl group with a cyclohexyl group has resulted in greater lipid solubility and increased permeability into normal mouse brain and tumor tissue (9). CCNU is slightly more effective than BCNU in prolonging life in mice with intracerebrally implanted L1210 leukemia (1). An additional advantage is its effectiveness when administered orally.

This study reports our clinical experience with CCNU in the therapy of carcinomas of the gastrointestinal tract, lymphoma, and a variety of other neoplasms.

MATERIALS AND METHODS

Fifty-nine patients whose ages ranged from 3 to 73 years were studied. Each patient had histologically proven malignancies that were not considered amenable to further therapy with surgery, irradiation, or the more conventional antineoplastic agents. Only five of the patients had not received previous treatment with X rays or chemotherapy. Eight patients had had X-ray therapy alone and 46 had received prior chemotherapy with or without previous radiation treatment. All of the patients with carcinoma of the gastrointestinal tract (stomach, pancreas, colon, or rectum) had previously received 5-fluorouracil (5-FU). Patients with lymphoma had been treated with either mechlorethamine or cyclophosphamide usually in combination with vincristine or vinblastine and prednisone (10,11). In all, alkylating agents (mechlorethamine, cyclophosphamide, chlorambucil, or melphalan) had been used in 23 patients and three others had received 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide (NSC–45388).

None of the patients, however, had received either cytotoxic drugs, corticosteroids, or X-ray therapy within 1 month of the initiation of treatment with CCNU. None had had prior treatment with another nitrosourea. All of the patients had measurable lesions that served as objective indicators of response. Responses were classified according to the evaluation system of Karnofsky (12). Within this system, complete remission was defined as the complete disappearance of all clinical evidence of disease, and partial response (favorable objective changes) was defined as a greater than 50% reduction in the sum of the products of the tumor diameters.

Hospitalization was required only when warranted by the severity of illness or when pretreatment evaluation was needed. For most patients, treatment was initiated and repeated as necessary in an outpatient clinic. Patients were usually seen at weekly intervals. Blood cell counts and tests of renal and hepatic function were obtained weekly. Response and toxicity were evaluated 6 weeks after the drug was started. Subsequent courses were then given at 6–8-week intervals to all patients who appeared to have responded or whose diseases had not shown definite progression of metastases.

CCNU, which was formulated in 40- and 100-mg capsules, was stored at −18 C until used. The drug was administered as a single oral dose at a level of 130 mg/m^2. This dose level was selected because a phase I study had shown it to be a safely tolerated maximum dose which produced dose-limiting but not cumulative toxicity when given at 6–8-week intervals (13). Antiemetics, usually prochlorperazine, were commonly given in an attempt to control nausea and vomiting.

RESULTS

The histologic diagnoses and responses to treatment for the entire series of patients are shown in table 1. Fifty-three of 59 cases were considered evaluable. Five patients died within 3 weeks of the initial therapy due to progressive disease, and serial studies did not adequately assess response in one other patient. A total of 119 courses of drug treatment were given. Responses were noted in 17 patients. Nine of these 17 responses were associated with a greater than 50% reduction in tumor masses which lasted a month or longer.

Lymphoma

Despite extensive prior drug therapy, the group of patients with lymphoma exhibited the best responses to CCNU observed in this study; six of ten patients demonstrated some response. While only subjective improvement was noted in three patients, objective antineoplastic responses occurred in three other patients. Two patients with Hodgkin's disease achieved complete remissions with durations which exceeded 9 and 13 months respectively. One patient with diffuse lymphoblastic lymphoma continues to respond after 4 months. The small number of patients in this group precludes analysis on the basis of histologic type, stage of disease, or prior treatment.

Gastrointestinal Cancer

The gastrointestinal cancer group had the largest number of patients treated. Twelve patients with colon cancer, two with rectal carcinoma, three with gastric cancer, and one with pancreatic carcinoma were studied. Only

TABLE 1.—Histologic diagnoses and therapeutic responses in patients treated with CCNU

Disease	No. of patients treated	No. of evaluable cases	No. of courses	Response category*				
				0–0	0–A	0–C	I–A	I–C
Carcinoma, colon	12	12	19	11			1(9)	
Hodgkin's disease	7	7	19	3	2(2,1.25)		1(9+)	1
Carcinoma, lung	6	5	9	3	1(2.5)	1(0.75)		
Brain tumor	4	3	20	1	1(4)		1(2+)	
Malignant melanoma	4	4	7	3			1(1.5)	
Non-Hodgkin's lymphoma	3	3	8	1	1(1)		1(4+)	
Carcinoma, cervix	3	3	7	2			1(4)	
Carcinoma, stomach	3	3	3	3				
Carcinoma, breast	3	2	4	2				
Carcinoma, head and neck	4	3	5	3				
Carcinoma, rectum	2	1	2	1				
Soft tissue sarcoma	2	2	6	1	1(6)			
Unknown primary carcinoma	2	1	2	1				
Hypernephroma	1	1	4		1(4)			
Ovarian teratoma	1	1	3				1(6)	
Plasma cell leukemia	1	1	2				1(2)	
Carcinoma, pancreas	1	1	1	1				
Total	59	53	119	36	7	1	8	1

*From Karnofsky (12): 0–0 = disease progresses without subjective or objective benefit; 0–A = subjective benefit without favorable objective changes; 0–C = subjective benefit and favorable objective changes of less than 1 month's duration; I–A = subjective benefit and favorable objective changes for more than 1 month; I–C = complete symptomatic improvement and regression of all manifestations of disease for more than 1 year. Numbers in parentheses represent duration of response in months.

one response was noted. This response was a very dramatic tumor regression in a 55-year-old woman with a 2-year history of metastatic cancer of the colon who had previously been treated with 5-FU and methotrexate. She had briefly responded to 5-FU and improvement had been sustained with methotrexate for 10 months before her condition again reached a moribund state with marked jaundice and hepatomegaly. She began to improve within 3 weeks after being treated with CCNU, with a marked reduction in liver size and improvement in liver function test results. The serum bilirubin level decreased from 30 mg/100 ml to normal in 8 weeks. Alkaline phosphatase values decreased from 920 to 212 IU and were maintained for 9 months before relapse. She died 1 year after CCNU treatment was begun. Second courses of CCNU were given without benefit to seven patients whose diseases had not clearly progressed after the first treatment. Some measure of the antecedent severity of the neoplastic process may be gauged by the overall survival of this group. One half of these patients died within 4 months after treatment with CCNU and no patients survived longer than 1 year.

Brain Tumors

Three of four patients with primary brain tumors demonstrated some drug effect. One 5-year-old child with an astrocytoma is stable and clinically well 22 months after drug treatment but is not clearly evaluable because of the lack of objective findings. Two of three adults with glioblastoma multiforme responded. One patient sustained subjective improvement for 4 months. Another was considered to have achieved objective tumor regression on the basis of marked improvement in his performance capabilities with clearing of mental confusion and diminution in what had been marked objective neurologic deficits. To date, this improvement has continued for 2 months.

Miscellaneous

Valuable objective antineoplastic effects were noted in four other patients. One 20-year-old woman with ovarian teratoma had a marked reduction in a pelvic mass for 6 months. One of three patients with carcinoma of the cervix experienced extensive reduction in supraclavicular node metastases which persisted for 4 months. One previously untreated patient with malignant melanoma responded for 2 months with regression of nodal and pulmonary involvement; three others with predominantly visceral disease unresponsive or now refractory to NSC-45388 failed to respond. One patient with acute plasma cell leukemia responded with clinical and hematologic improvement characterized by a rise in platelet count and a disappearance of blast forms from the peripheral blood.

Toxic Effects

In general, CCNU was well tolerated symptomatically. Mild nausea and vomiting usually occurred 2–6 hours after the drug was administered and rarely lasted more than 4 hours. The concomitant and sometimes prophylactic use of antiemetics prevented an accurate assessment of the frequency and intensity of these symptoms; most of these reactions occurred at home after out-patient administration of the drug. In no case did nausea and vomiting result in dehydration, prevent patient acceptance of a second course of therapy, or necessitate hospitalization. No renal, hepatic, or central nervous system toxicity was encountered which was not explained clinically by progressive metastatic disease and subsequently documented by post-mortem examination. Hematologic toxicity was the major complication and limited the use of the drug. Mild anemia, granulocytopenia, and thrombocytopenia were common with nadir values for all elements generally occurring 24–26 days after treatment, though the nadir developed as early as 7 and as late as 49 days after treatment (table 2). The influence of pretreatment hematologic values on the extent of subsequent toxicity was examined. While antecedent anemia increased the number of patients with hematocrit nadirs below 25%, it did not affect the degree of granulocytopenia or thrombocytopenia. Profound thrombocytopenia developed in all five patients with pretreatment abnormal white blood cell or platelet counts; three of these had known marrow involvement established by biopsy (table 3). Prior chemotherapy did increase the degree of thrombocytopenia observed. Platelet nadirs below 50,000 platelets/mm^3 occurred in three of

33 courses (9%) in patients who had had no prior chemotherapy; in contrast, this degree of thrombocytopenia developed in 20 of 75 courses in previously treated patients (27%) (table 4). Toxic effects in subsequent treatments were no more severe than in the initial course. Despite this frequency of thrombocytopenia, no significant bleeding episodes occurred. The one drug-related death in this study resulted from gram-negative septicemia in a severely ill patient with drug-resistant Hodgkin's disease and marked granulocytopenia.

DISCUSSION

The mechanism of action of the nitrosoureas remains obscure. While substitution of a cyclohexyl group for a chloroethyl moiety may have altered alkylation (14) and increased protein binding (15), it has not affected the prolongation of DNA synthesis produced by these compounds (16,17). Clinically, CCNU retains the unique delayed hematologic toxicity and the spectrum of antineoplastic activity noted with BCNU (5).

TABLE 2.—Hematologic suppression after initial course of CCNU therapy

	Nadir value		Nadir day	
	Median	Range	Median	Range
Fall in hematocrit value (%)	4	0–22	24	7–41
White blood cell count ($\times 10^3$/mm^3)				
Total	3.9	15.0–0.2	26	7–49
Granulocyte count	2.9	12.0–0.1	—	—
Platelet count ($\times 10^3$/mm^3)	140	490–2	25	7–49

TABLE 3.—Effect of pretreatment hematologic status on toxicity after initial course of CCNU therapy

Pretreatment hematologic status*	No. of evaluable cases/No. treated	Hematocrit nadir (%)			Granulocyte nadir (mm^3)		Platelet nadir ($\times 10^3$/mm^3)	
		35–31	30–25	<25	1500–750	<750	100–50	<50
Normal	27/32	4	7	2	5	2	10	3
Anemic	20/22	7	6	7	3	3	2	4
Abnormal	5/5		2	3	1	2		5

*Normal = hematocrit value, >35%; white blood cell count, >5000 cells/mm^3; and platelet count, >150,000 platelets/mm^3. Anemic = hematocrit value, <35% as the only abnormality. Abnormal = reduced white blood cell and/or platelet count values irrespective of hematocrit values.

TABLE 4.—Effect of prior chemotherapy on hematologic toxicity after CCNU therapy

	No. of evaluable courses	Hematocrit nadir (%)			Granulocyte nadir (mm^3)		Platelet nadir ($\times 10^3$/mm^3)	
		35–31	30–25	<25	1500–750	<750	100–50	<50
No prior chemotherapy								
Initial treatment	13	2	3	3	2	3	4	2
Subsequent courses	20	2	3		2		1	1
Prior chemotherapy								
Initial treatment	39	9	12	9	7	4	8	10
Subsequent courses	36	10	4	3	7	2	4	10

In this study, objective antineoplastic responses were seen in lymphoma, brain tumors, malignant melanoma, ovarian teratoma, plasma cell leukemia, and carcinoma of the colon and cervix. The small number of patients within most tumor types precludes a comparison with other concurrent studies of CCNU or even our previous study with BCNU. With the possible exception of our experience in brain tumors where two of three patients with glioblastomas responded, these results do not support claims to therapeutic advantage for CCNU. In gastrointestinal cancer, the largest group of neoplasms in the study, improved response rates were not realized. These discouraging results are similar to those cited by Moertel et al (18, 19) and do not support the continued study of this agent in gastrointestinal cancer. In Hodgkin's disease, only two of seven patients responded. This response rate is comparable to those rates previously achieved with BCNU (6–8) and is not as favorable as described for CCNU by Selawry and Hansen (20).

CCNU may have several advantages in terms of toxicity. The oral administration of the nitrosourea is convenient and avoids the flushing and arm pain produced by the intravenous infusion of BCNU. More important, 130-mg/m^2 dose levels of CCNU may be better tolerated than 150–200-mg/m^2 dose levels of BCNU. In their study, Hansen et al (13) noted that the nadir of leukopenia occurred on Day 38, some 9–10 days after the nadir of thrombocytopenia. In our study, leukopenia occurred earlier and generally coincided with thrombocytopenia on about the 25th or 26th day. Platelet count nadirs below 50,000 platelets/mm^3 developed in 23% of all patients in this study, compared to 34% in our previous study with BCNU (8). More significantly, bleeding complications contributing to death occurred in two to five patients in that study and none were seen in this study. Furthermore, subsequent courses did not produce additive hematologic effects.

More recently, clinical trials with another nitrosourea, methyl-CCNU, have begun based on the superior effects of this derivative against metastatic Lewis lung carcinoma (21). Methyl-CCNU retains the hematologic suppressant characteristics of the nitrosoureas (22,23) but may also produce serious central nervous system toxicity.[6]

Further studies will be necessary to define the role of this class of compounds in the chemotherapeutic treatment of cancer.

LIST OF COMPOUNDS

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; butyric acid, 4-[*p*-[bis(2-chloroethyl)amino]phenyl]-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride; HN_2

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

1-Methyl-1-nitrosourea: NSC-23909; CAS reg. No. 684-93-5

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-; DTIC; imidazole carboxamide

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Phase I Study of Tilorone, a Non-Marrow Depressing Antitumor Agent [1,2,3]

G. L. Wampler, M. Kuperminc, and W. Regelson [4]

SUMMARY

In preclinical studies tilorone has been shown to induce interferon, to protect mice from lethal virus and staphylococcal infection, to stimulate phagocytic response, to enhance 19S antibody production, and to inhibit a variety of transplanted and virus-induced tumors without demonstrable inhibition of bone marrow function. For this reason, tilorone was entered into clinical study. In a phase I study, tilorone produced partial responses in two patients with metastatic melanoma who were previously untreated by chemotherapy. Treatment is continuing in both patients. Of ten patients who received therapy for 2 weeks or longer, eight had measurable disease: two of these patients showed partial responses, and in six patients there was "no change." Tilorone does not depress the bone marrow function and, on the contrary, may stimulate platelet production. Of interest, basophilic inclusions similar to those reported in animals were seen in leukocytes. The primary toxic effect of tilorone is lethargy, but nausea and vomiting, weakness, anorexia, diarrhea, belching, dizziness, headache, and insomnia have been seen at higher dosage levels. Because of tilorone's sedative and/or emetic action at higher dosages, the maximum daily tolerated dose suggested by this study is 10 mg/kg/day.

[Cancer Chemother Rep 57:209–217, 1973]

Tilorone (fig 1) stimulates phagocytic response (1,2), enhances 19S antibody production (2),[5] and inhibits a variety of transplanted and virus-induced tumors (2,3–5). It induces interferon (6–10) and protects mice from lethal viral (6–10) and staphylococcal infection (1,2).

Because of its potential antiviral and/or antitumor activity in man, tilorone was administered in a phase I study to 15 patients between 16 and 73 years of age. All patients entered into this study had pathologically proven

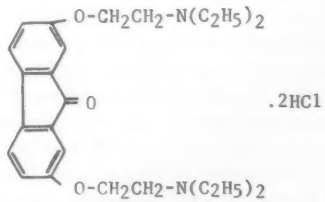

FIGURE 1.—Structure of tilorone.

[1] Received Dec 26, 1972; revised Apr 16, 1973.

[2] Supported by grants from the Eastern Cooperative Oncology Group, Merrell-National Laboratories, and the Irwin Strasburger Memorial Medical Foundation.

[3] Tilorone: NSC-143969; fluoren-9-one, 2,7-bis[2-(diethylamino)ethoxy]-, dihydrochloride.

[4] Division of Medical Oncology, Medical College of Virginia, Virginia Commonwealth University, Richmond.

[5] Hoffman PF, Ritter HW, and Krueger RF. Tilorone hydrochloride stimulation of the primary immune response of mice. Presented at the 7th International Congress of Chemotherapy, Prague, Austria, Aug, 1971.

cancer beyond the hope of cure by surgery or radiation therapy. There were four patients with carcinoma of the ovary, three with malignant melanoma, two with synovial sarcoma, and two with carcinoma of the pancreas (one of these carcinomas was a gastrin-secreting islet cell tumor). One patient each had carcinoma of the tonsil, carcinoma of the breast, cylindroma, and lymphosarcoma. Five patients were not adequately studied: four of these patients received the medication for 3 days or less and a fifth patient died on the eighth day of treatment. The remaining ten patients took the drug for 2 weeks or longer. Each of eight patients received a total cumulative dose of 10–22.5 g of tilorone (140–360 mg/kg).

Tilorone was given orally in divided dosage. Based on animal data and a previous single-dose treatment in volunteers, the initial dosage selected was 4 mg/kg/day. After two patients had received treatment at this level, the dose was increased to 6 mg/kg/day for an additional two patients, then to 8 mg/kg/day for another two patients, and finally to 10 mg/kg/day for a final five patients. A single patient received a dose of 12 mg/kg/day which had to be discontinued after 3 days because of severe nausea and vomiting unrelieved by phenothiazine and antiemetics.

RESULTS

Toxic Effects

Side effects were common but were not serious. Most of the patients complained of one or more effects reflecting central nervous system (CNS) or gastrointestinal (GI) toxicity. At the lower dose levels of 4 and 6 mg/kg/day, side effects were milder but persistent. The most frequent complaint was drowsiness or sedation, reported by six of the patients, which typically started about ½ hour after each dose of tilorone. Sedation lasted a few minutes to several hours depending on the severity of the side effect. Two ambulatory patients treated at the 10-mg/kg dose level were forced to spend a good deal of time in bed due to this symptom.

Other CNS toxic effects reported were episodes of headache, dizziness, abnormal dreams, insomnia, transient diplopia, and a feeling of "explosion in the head." Most of these side effects, however, were reported by a single patient treated at 10 mg/kg/day and were inconstant in their appearance during his 2 weeks of treatment.

Nausea occurred in five of 15 patients and those with the most severe nausea also reported occasional vomiting. Several patients complained of loss of appetite. Diarrhea occurred in three of 15 patients and in two of these it appeared to be clearly drug related. The two patients with carcinoma of the pancreas had had diarrhea prior to starting treatment with tilorone, but in one the symptoms were definitely aggravated by tilorone. Other GI symptoms reported were dryness of the mouth and annoying belching or barborygmi in one patient each. Generally, these symptoms lasted up to 1 week after tilorone was discontinued.

A skin rash developed in one patient while he was receiving tilorone. The rash initially resembled seborrheic dermatitis and persisted for over 2 months after therapy was stopped (to the time of this writing). We feel the rash is probably not drug related.

Table 1 lists the side effects noted in each patient.

Laboratory Data

Hemoglobin.—No changes in hemoglobin level were noted.

Leukocytes.—Figure 2 graphs the white blood cell (wbc) counts of nine of the 12 patients receiving a total of 1 or more g of tilorone. It can be seen from this that no significant depressing effect on the total wbc count by tilorone was apparent. If anything, leukocytosis was seen and more data must be obtained to confirm this. No effect on the differential count was noted but "toxic granulations" were reported frequently in peripheral smears, and inspection of the smears indicated the presence of basophilic granules in leukocytes (fig 3) which were the same as those reported in the preclinical animal studies (3).[6] Bone marrow examinations also showed these

[6] Hook PH. Personal communication, Merrell-National Laboratories, Cincinnati, Ohio, 1971.

TABLE 1.—Tilorone dosage and side effects

Patient No.	Age (yrs)	Tumor	Tilorone		Total cumulative dose		Therapy changes	No. of days of therapy	Side effects
			Mg/kg/day	Total dose/day (mg)	Mg/kg	Grams			
1	50	Ovary				0.5	Therapy stopped	1	Nausea and vomiting
2	16	Lymphosarcoma				0.25	Therapy stopped	1	Excessive drowsiness
3	52	Ovary		150		0.3	Patient died	2	None recorded
4	48	Ovary	4	250	264	16.5	Received 2 courses only	66	Nausea, less active
5	53	Breast	4	250	272	17	Therapy stopped for 1 wk	63	Slight nausea and drowsiness
6	69	Islet cell, pancreas	6	375	84	5.25	Therapy stopped twice	14	Lack of appetite, weakness, diarrhea†
7	65	Pancreas	7	250	322	11.5	No changes	46	Diarrhea†
8	62	Ovary	8	400	360	18.0	Received 2 courses only	45	CNS depression (?)
9	37	Melanoma	8	750	240+	22.5+	No changes	30+	Headache, dizziness
10	54	Melanoma	10	500	80	4	Patient died	8	CNS depression (?)
11	49	Cylindroma	10	750	140	10.5	No changes	14	Diarrhea, drowsiness, abnormal dreaming (?), decreased appetite, belching, nausea
12	69	Tonsil	10*	675*	40	2.55	Patient did not take full dose	14	Drunk feeling, sleepiness, dry mouth, skin rash (drug-related ?)
13	59	Melanoma	10	500	350+	17.5+	No changes	35+	None recorded
14	39	Synovial sarcoma	10	750	140	10.5	No changes	14	Transient diplopia, insomnia
15	73	Synovial sarcoma	12	500	36	1.5	Therapy stopped	3	Severe nausea and vomiting

*Prescribed dose was not taken.
† Diarrhea was present before tilorone therapy was started.

basophilic granules in the mature leukocytes or late leukocyte precursors.

Platelets.—Tilorone appears to have a rapid and fairly predictable initial stimulatory effect on the platelet count (*see* fig 4). In the first week of therapy, more than one half of our patients had an increase in the platelet count which was often two or more times greater than the baseline value. Thereafter, there seemed to be a gradual decline in the platelet count toward baseline values even though the drug was still being administered and the body concentration of the drug was presumably increasing. Tilorone therapy was restarted in two patients after they had not received the drug for 5 and 21 days respectively, and each time a second stimulation of the platelet count was observed.

Two patients who had had thrombocytopenia as a result of previous chemotherapy, and in whom thrombocytopenia developed during or after tilorone therapy, had bone marrow examinations when the peripheral platelet count was 60,000 cells/mm³ or less. One of these patients (No. 4) had an initial rise in the platelet count to over 200,000 cells/mm³, but despite continued tilorone administration a secondary fall occurred. The bone marrow examination was done on Day 35 at which time she was still receiving tilorone at the same dose of 250 mg/day (6 mg/kg/day). The other patient (No. 12) had been receiving tilorone for 2 weeks and had received a low dose averaging less than 4 mg/kg/day (2.55 g total dose). The platelet count gradually fell from 112,000 to 60,000 cells/mm³ by Day 42. The bone marrows of both these patients showed the presence of adequate megakaryocytes. The reason for the low peripheral counts appeared to be diminished platelet production. It is interesting that

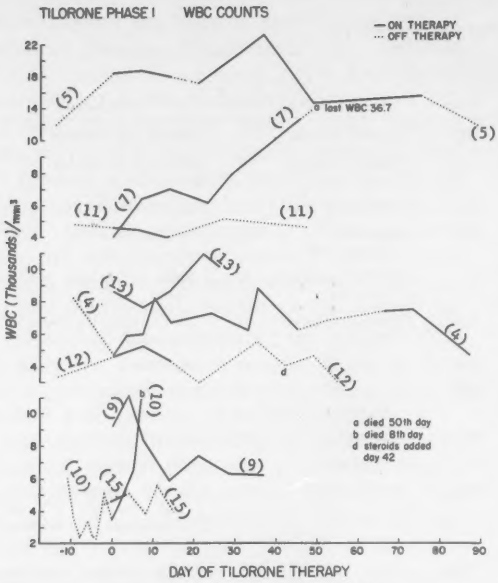

FIGURE 2.—Wbc counts in 9 of the 12 patients receiving a total of 1 or more g of tilorone. Numbers in parentheses are patient Nos.

one of these patients (No. 12) was then given prednisone at a dose of 30 mg/day and within 1 week the platelet count was restored to normal (272,000 cells/mm³).[7]

Electroencephalogram.—Four patients exhibiting CNS toxicity of varying degrees had normal EEGs. These tests were done at a time when three of the patients were symptomatic with lethargy and one patient had transient diplopia and "bizarre feelings."

Total protein.—About one half of the patients showed slight increases in total protein while receiving tilorone. At least three had elevations of total protein above the normal range, with serum electrophoresis in one of these patients showing a generalized increase in total gamma-globulin. These fluctuations in blood proteins may not be significant but are worthy of further investigation.

Liver function.—No evidence of functional liver damage was present. Bilirubin, serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase, cholesterol, and lactic dehydrogenase (LDH) determinations were done in all patients. One patient had an elevated alkaline phosphatase level to 800 microunits/ml on Day 3 of therapy which thereafter returned to normal (after four subsequent determinations). One can only speculate, until more patients are studied, that if the alkaline phosphatase level of 800 microunits/ml was valid it may have been due to a mechanism other than liver damage. Several patients had significant decreases in SGOT and/or LDH levels presumably related to tumor effects rather than changes in liver cell metabolism.

Kidney function.—No change in blood urea nitrogen or urine output was noted.

Other serum chemistry tests.—No significant changes in calcium phosphorus, glucose, or uric acid levels were noted.

TUMOR EFFECTS

Of the ten patients receiving therapy for 14 days or longer, eight had measurable or evaluable disease. Two partial responses were noted, one of which is continuing as of this writing, 7 months from the start of the study. In both partial responses, metastatic melanoma was the diagnosis and the patients had been previously untreated with other chemotherapy. Figure 5 shows the chest roentgenograms of one of the responders (No. 9). The patient had been receiving therapy for 3 weeks at the time the partial response was documented. Subsequent chest films taken at 6 and 12 weeks show further resolution of the mediastinal lymph nodes with stabilization of changes seen 6 months after treatment. The other responder had questionable brain metastases with apathy and inability to function normally. In addition, palpable diffuse metastatic disease in the left breast and chest wall was present. For 1 month the patient exhibited a clearing of her mental depression, with return to full household function, and an ability to drive her car. The disease had virtually disappeared in the left breast and chest wall. Tumor persisted in the left axilla, and all tumor areas showed recurrent growth 3 months later despite continued tilorone therapy. Two weeks after tilorone therapy was discontinued,

[7] Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1, 4-diene-3,11,20-trione, 17,21-dihydroxy-.

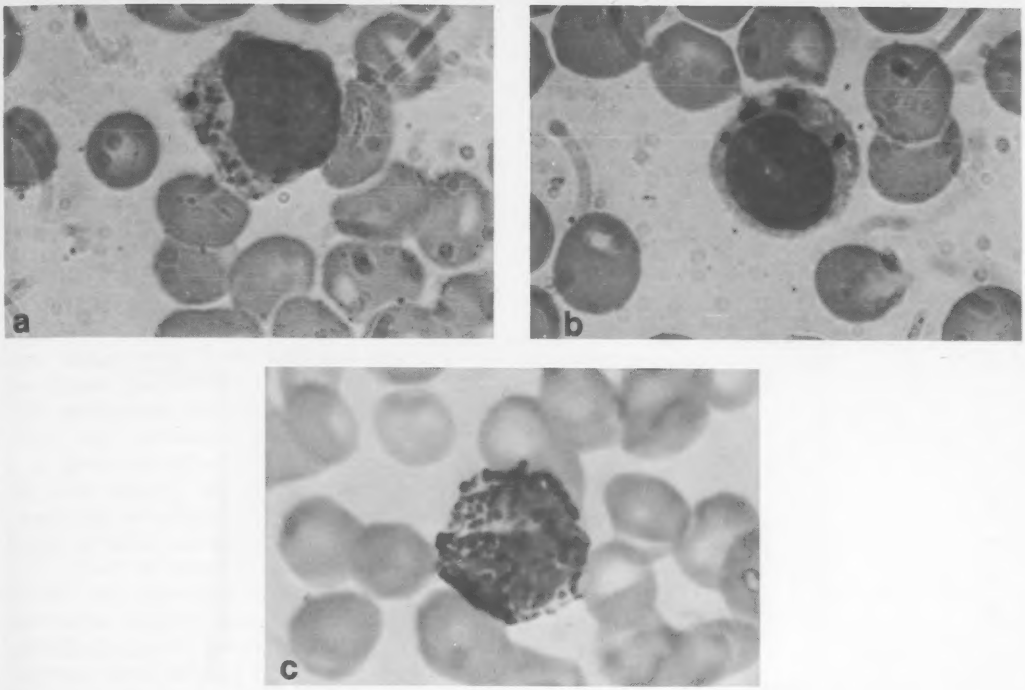

FIGURE 3.—Typical basophilic cytoplasmic inclusions in lymphocytes and monocytes.

Bacillus Calmette-Guérin (BCG) was given intracutaneously. Tumor necrotization occurred and the patient died of her disease 4 months after the tilorone treatment was stopped. She had also been given 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388; CAS reg. No. 4342-03-4) with no therapeutic effect.

A third patient (with cylindroma) showed a return to normal of SGOT, LDH, and alkaline phosphatase levels which had been elevated for 4 months. These enzyme levels remained normal 2 months after tilorone was discontinued. The metastatic lesions have not changed in size after 6 months.

DISCUSSION

In this phase I study a rather consistent initial stimulation of the platelet count was seen, but the count showed a tendency to fall later in spite of the fact that the concentration of the drug in tissue or tissue fluids was presumably increasing. This assumption seems reasonable in view of a demonstrated 3.8-day half-life in mice and what may be an even longer half-life of tilorone in man.[8] Based on our initial experience, it may be that after further clinical trials a delayed effect of tilorone may result in a later fall in platelet count reflecting the exhaustion of the original stimulating mechanism. Thus, the stimulation of the platelet count by tilorone appears to resemble that seen for interferon induction, namely stimulation followed by exhaustion of response on repeated drug administration.

Prolonged administration of tilorone at 10 mg/kg/day in monkeys produced ptosis and tremor. At higher levels (20 mg/kg/day) sudden death associated with cerebral edema was seen in four monkeys between 21 and 23 days

[8] *See* footnote 6.

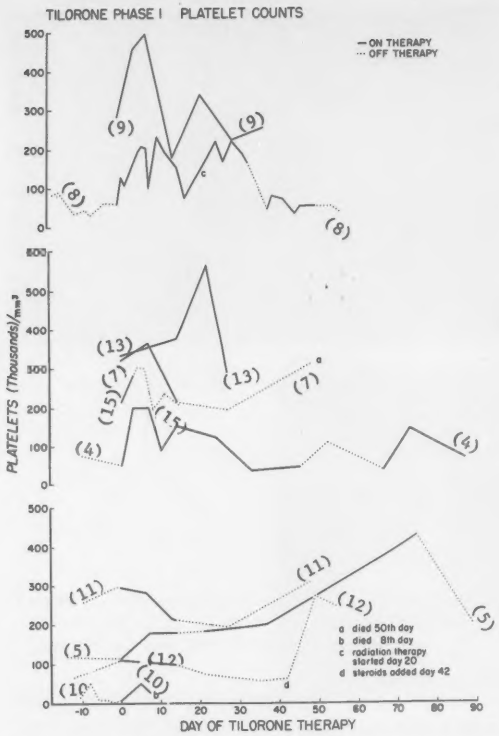
TILORONE PHASE I PLATELET COUNTS
— ON THERAPY
··· OFF THERAPY
PLATELETS (Thousands/mm³)
DAY OF TILORONE THERAPY
a died 50th day
b died 8th day
c radiation therapy started day 20
d steroids added day 42

FIGURE 4.—Platelet counts in 10 patients receiving tilorone. Numbers in parentheses are patient Nos.

after the treatment was started.[9] For this reason, CNS toxicity was looked for in our clinical study but the only significant toxicity was readily reversible CNS depression or sedation. These side effects in man were apparently related to peak plasma levels of the drug[10] as marked sedation was seen after the first dose of tilorone and this sedation usually occurred about ½ hour after each dose in those patients reporting the symptom. The effect lasted from a few minutes to several hours. In one patient who reported excessive sedation after a 225-mg tid dose, satisfactory control of this annoying drug-related symptom was achieved during the day by giving 150 mg tid with 225 mg at bedtime. Similarly, the one reported case of headache occurred transiently after each dose and could be controlled by decreasing the dose. Paradoxically, insomnia was reported in one patient but this disappeared despite maintenance of the same dosage after 1 week. Neither insomnia nor somnolence was related to any other medication that these patients were taking.

Although looked for, we did not note cumulative toxicity. In view of the purported long biologic half-life of the drug,[8,10] this type of effect was originally presumed to be the dose-limiting problem that would be of major concern. This related to the previously mentioned studies in rhesus monkeys who were given 450–500 mg/kg as a total cumulative dose (10). In these monkeys four deaths occurred in a cluster suggesting that the deaths were indeed caused by the total cumulative dose; however, autopsy of these animals failed to reveal a well-defined histologic cause of death. Fortunately, in our study, although side effects that were initially present increased somewhat in severity with continued drug administration, this did not limit further drug administration. In general, the pattern of side effects seen in individual patients was clearly obvious within 24 hours to 1 week after tilorone therapy was started. The effects did not change appreciably with continued drug administration.

The fourth type of tilorone effect looked for was an effect on the immune response, such that the course of disease or toxicity related to immunity could develop for a duration beyond the actual presence of drug. The skin rash seen in one patient could represent this type of phenomenon since it developed while the patient was receiving treatment and continued to be present 2 months later. Structural resemblance to phenothiazines or tricyclic antidepressants which have a high affinity for melanin suggest that skin sensitization to light is an effect that must be looked for although it does not appear to be the case in this one patient.

In relation to possible stimulation of latent host responses, the patient with cylindroma had had elevations of SGOT (190–350 microunits/

[9] Tilorone Pre-clinical Toxicology Report, Merrell-National Laboratories, Cincinnati, Ohio, 1971.

[10] Wright G. Personal communication, Merrell-National Laboratories, Cincinnati, Ohio, 1972.

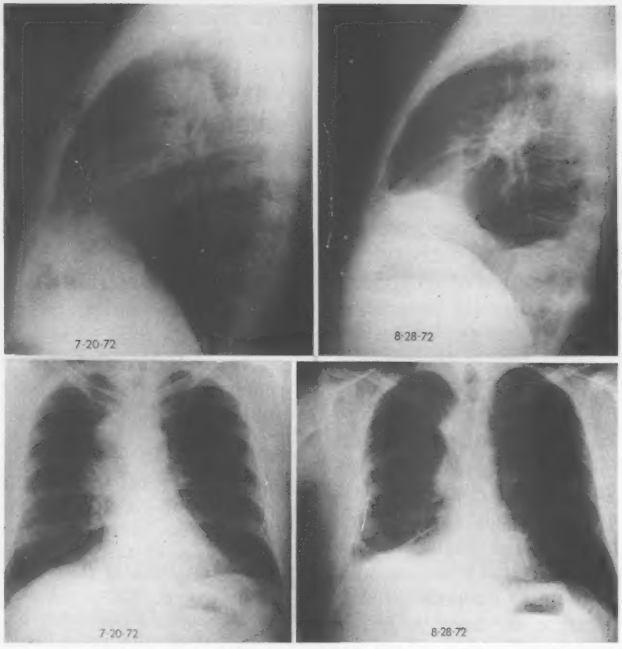

FIGURE 5.—Regression of metastatic melanoma lesion. Patient is asymptomatic and regression persists at this same level 6 months later.

ml), LDH (250–325 microunits/ml), and alkaline phosphatase (90–135 microunits/ml) for 4 months (five determinations) prior to starting tilorone therapy. Six days after tilorone therapy was begun, all these values were normal and remained normal 2 months after tilorone was stopped.

At this time it is not possible to assess accurately the effect tilorone has on tumors. In our animal studies there was some evidence that the effect may be cumulative, but this is not definite and needs further investigation.

While tilorone has been reported to stimulate circulating antibody response as measured by Jerne plaquing (2),[11] in our hands it prolongs skin graft survival [12] and thus may inhibit some aspects of cellular immunity. In fact, in a system utilizing a mouse sarcoma virus-derived tumor, tilorone can, like other interferon inducers (eg, poly IC [11]), promote tumor growth (12).[13] This effect may relate to the time of drug administration (12).[14]

In the clinical study by Kaufman et al (13) on interferon induction, dosage was not reported on an mg/kg basis nor was the dose/timing or character of the buffer or vehicle used reported. The only side effects indicated by Kaufman et al in their study were "gastrointestinal upset with a little diarrhea and in one case vomiting."

Tilorone represents a new series of relatively nontoxic antitumor agents which do not suppress marrow function. The presence of cytoplasmic inclusions in leukocytes and phagocytic reticuloendothelial cells has been reported

[11] *See* footnote 5.

[12] Munson AE, and Regelson W. Work in progress, 1972.

[13] Poly IC: NSC-120949; 5'-inosinic acid, polymers, complex with 5'-cytidylic acid polymers (1:1); polyinosinic:polycytidylic acid.

[14] Gazdar AF. Personal communication, 1972.

for the synthetic polyanion, pyran copolymer, which also possesses phagocytic-stimulating, interferon-inducing, and antitumor properties (14).[15] These cytoplasmic inclusions do not relate to toxicity, and in animal studies cytoplasmic inclusions can be seen in the absence of interferon induction or antiviral effect for structures related to tilorone.[16]

As mentioned previously, tilorone possesses interferon-inducing and antiviral activity. The antiviral activity for this series of compounds does not necessarily relate directly to levels of circulating interferon. Tilorone stimulates phagocytosis, and related congeners of tilorone can protect mice against lethal staphylococcal infection during the period of phagocytic stimulation (2,15). Tilorone has antitumor activity against a variety of transplanted tumors and when administered in drinking water can inhibit L1210 leukemia (15).[17] Tilorone represents the first of a new series of orally and parenterally active antitumor and antiviral agents as demonstrated by animal data (2,15),[17] and to our knowledge it is the only orally administered agent which can stimulate phagocytic response in mice (1,2).

In relation to the clinical action of tilorone, we have developed some evidence that schedules of administration in animals can influence toxic effects (15). Gazdar had shown that this could be pertinent to the paradoxic promotion of tumor growth (11,12).[18] In this regard, the exploration of various drug schedules is essential, and it should also be noted that interferon response can be lost after daily administration in an animal system (10).

More work must be done to establish the validity and consistency of the observation that tilorone transiently reverses the late effects of thrombocytopenia secondary to tumor effects and/or chemotherapy. If valid, one might expect that intermittent dosage schedules related to antiviral schedules in mice might be more effective than daily antitumor schedules.

Further clinical investigations of this interesting compound and eventually related congeners (2) [17] is necessary, because these structures represent a new departure from previous antitumor substances that stimulate host response. Tilorone is distinctly unlike BCG, *Corynebacterium parvum*, poly I:C, methanol extract residual (MER), and pyran copolymer since it is orally as well as parenterally active. Tilorone is now ready for phase II study and it will be of interest to see if it continues to show clinical antitumor activity against melanoma or other tumors.

[15] Pyran copolymer: NSC-46015; pyran-2-succinic anhydride, 4,5-dicarboxytetrahydro-6-methyl-, anhydride, polymers.

[16] Mayers GD. Personal communication, Merrell-National Laboratories, Cincinnati, Ohio, 1972.

[17] Wampler GL, Munson AE, and Regelson W. Tilorone and congeners increase in murine life span with Friend virus leukemia and L1210 leukemia by chronic oral administration. Submitted for publication.

[18] *See* footnote 14.

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Kinetic Scheduling of Vincristine (NSC-67574) and Bleomycin (NSC-125066) in Patients With Lung Cancer and Other Malignant Tumors[1,2,3]

R. B. Livingston, G. P. Bodey, J. A. Gottlieb, and E. Frei III [4]

SUMMARY

Forty-eight patients received vincristine and bleomycin given in sequential combination. Of 32 evaluable patients, nine had a partial response (28%), including four of 15 with lung cancer (27%) and three of nine with head and neck cancer (33%). The results of sequential combination therapy in lung cancer appear to be superior to those previously reported for bleomycin alone. Toxicity of the combination was no greater than that expected of each agent. There was no significant myelosuppression. Further trials of this combination, and exploration of additional combinations incorporating myelosuppressive agents, appear warranted.

[Cancer Chemother Rep 57:219–224, 1973]

Bleomycin is an antitumor antibiotic which has recently received extensive trials as a single agent. It has a broad spectrum of activity against human neoplasms, particularly squamous cell carcinoma (1,2) and lymphomas (3), and possesses very little myelosuppressive activity. In spite of its reported activity in squamous cell cancer, and the fact that it concentrates highly in lung tissue, evaluation of bleomycin in Europe (2,4) and in the United States has revealed a disappointing response rate in lung cancer. Sixty-eight patients with that diagnosis participated in a study of bleomycin conducted by the Southwest Cancer Chemotherapy Study Group, the largest reported series (5). Only three of 60 evaluable patients (5%) had a greater than 50% tumor regression. In this study, the drug was given either intravenously (iv) or intramuscularly (im), at a dose of 10 mg/m^2 of body surface area, twice each week.

Barranco and Humphrey (6) have recently demonstrated that bleomycin is most effective at killing Chinese hamster cells in vitro when the cells are in mitosis and G_2 phase, with mitosis the more sensitive. A concentration of 1 μg/ml for 30 minutes killed 60% of the cells in mitosis and 50% of the cells in G_2 phase. Cells in G_1 phase and S phase were less sensitive and responded only to a much higher concentration of the drug.

The vinca alkaloids have shown a striking ability to increase temporarily the population of cells in mitosis, both for human bone marrow and for solid tumors in man (7,8), with maximal effects apparent in 6–12 hours after administration of the drug. Experimentally, the

[1] Received Dec 29, 1972; revised Mar 9, 1973.

[2] Supported by grants CA-05831, CA-10379, and CA-03754 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Vincristine: CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1). Vincristine and bleomycin were supplied by the Cancer Therapy Evaluation Branch, Division of Cancer Treatment, NCI.

[4] Department of Developmental Therapeutics, The University of Texas at Houston, M.D. Anderson Hospital and Tumor Institute, Texas Medical Center.

disruption of the mitotic spindle is reversible (9), and the lack of concordance between the stathmokinetic and cellular destructive effects of vincristine (as in normal human bone marrow) implies that at least some cells which are temporarily arrested in mitosis later "escape" and successfully divide.

Vincristine alone has not received an adequate trial in the treatment of lung cancer (10); however, some activity in oat cell carcinoma has been reported (11). It has also been reported in the English-language literature (10) that vinblastine [5] used alone was reported to produce objective tumor regression in 11% of 109 patients with lung cancer.

In vivo data supporting sequential administration of vincristine and bleomycin were provided by an experiment in the Lewis lung tumor. In this slow-growing animal model system, Schabel [6] found that, although vincristine itself had no antitumor effect, prior treatment with vincristine enhanced the antitumor activity of bleomycin.

Based on the foregoing considerations, a protocol was devised for the treatment of patients with advanced metastatic cancer, using vincristine and bleomycin in sequential combination in an attempt to synchronize tumor cells with vincristine for subsequent destruction by bleomycin. It was postulated that 6–12 hours after vincristine was given the number of tumor cells in mitosis and G_2 phase would be maximal. Therefore, bleomycin was given 6 hours after vincristine, when it would presumably be most effective.

METHODS

Patients with inoperable or metastatic solid tumors, especially those with primary lung tumors, were eligible for study. Patients who had received prior therapy with bleomycin or vincristine were considered ineligible for the study. Baseline spirometry tests, determinations of blood gases, and chest roentgenograms were performed on all patients, and those with an arterial oxygen tension (partial pressure of O_2) less than 70 mm or a total or one-second vital capacity less than 50% of the predicted value were excluded from study.

Vincristine was given in a dose of 0.75 mg for patients 50 years of age or older and 1 mg for those under 50. A rapid iv injection was administered twice weekly in the morning. Six hours after each dose of vincristine, 30 mg of bleomycin was given, usually iv, in 50 ml of 5% dextrose in water; some patients received the drug im because of technical difficulties in iv administration. The twice-weekly injections were continued for a total of 12 doses (6 weeks). At the end of this period, the drugs were discontinued. The investigator then had the option of giving the patient other chemotherapy or of observing the patient until his disease progressed. The total dose of bleomycin administered to a patient during a full course of therapy was 360 mg. Bleomycin was discontinued earlier if evidence of pulmonary toxicity developed; the therapy was withheld if stomatitis or skin ulceration occurred and was not restarted until that manifestation had subsided. Vincristine was discontinued if evidence of severe neuropathy developed, as defined by foot drop, severe abdominal pain, inability to button clothes or to write, or obstipation.

Patients were evaluated while receiving therapy with a weekly complete blood cell count, including differential and platelet counts. Pulmonary function tests were repeated at 4 weeks and, whenever possible, subsequently at 4-week intervals.

Partial response was defined as a greater than 50% reduction in the sum of the tumor areas determined by multiplying the two greatest diameters of all measurable tumors, without an increase in any mass or the appearance of new lesions. Patients were evaluable for adequate trial if they received at least 2 weeks of therapy (four injections). They were considered to have progressive disease when a greater than 50% increase in tumor size was observed, and at that point they were removed from the study. No patient was removed from the study before the end of 6 weeks unless definite evidence of progression had taken place.

RESULTS

Antitumor Efficacy

Forty-eight patients were entered into the

[5] Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate.

[6] Personal communication.

study. Twelve patients were not evaluable for either toxic effects or response, because of death related to tumor within 2 weeks of the start of the study (nine patients) or inadequate follow-up (three patients). Four additional patients were evaluable for toxic effects but were not evaluable for response, because they received additional antineoplastic drugs (two), were ineligible (one), or had no evaluable disease (one). The response and toxicity data are detailed in table 1.

Of 17 patients with lung cancer who were

TABLE 1.—Response and toxicity in 36 evaluable patients

Patient No.	Diagnosis	Toxicity	Response: comment*
1	Lung (squamous)	Fever	NR
2	Lung (adenocarcinoma)	Skin (mild)	PR: regression of lung masses, roentgenogram showed no change in pelvic disease
3	Lung (squamous)	Skin (moderate)	<50% regression
4	Lung (oat cell)	Skin (mild)	PR: regression of lung mass
5	Lung (adenocarcinoma)	Skin (mild), neuropathy (moderate)	NR
6	Lung (adenocarcinoma)	Neuropathy (?)	NR: removed from study at 4 wks with myocardial infarction
7	Lung (squamous)	Pulmonary (nonfatal, severe)	PR: >50% regression of lung lesion, no change in skin nodules
8	Lung (adenocarcinoma)	Pulmonary (?) ("diffuse inflammatory process") and death at another hospital (age 69)	<50% regression
9	Lung (adenocarcinoma)	Neuropathy (moderate)	PR: response in lung and liver metastases
10	Lung (adenocarcinoma)	Neuropathy (mild)	NR
11	Lung (adenocarcinoma)	None	NR
12	Lung (adenocarcinoma)	Skin (mild)	PD
13	Lung (squamous)	Thrombocytopenia (? due to drugs)	<50% regression
14	Lung (squamous)		NR
15	Lung (adenocarcinoma)	Pulmonary (?), slight reduction in vital capacity, no clinical problem	PR: also received 5-FU† (inevaluable); progressed rapidly on 5-FU maintenance after bleomycin and vincristine discontinued
16	Lung (adenocarcinoma)	None	PD: six doses, dose reduced because of age
17	Nasopharynx (squamous)	Pulmonary (?) associated with pneumonia	PD: transient improvement early

TABLE 1.—Response and toxicity in 36 evaluable patients—Continued

Patient No.	Diagnosis	Toxicity	Response: comment*
18	Nasopharynx (squamous)	Skin (moderate), neuropathy (moderate)	PD
19	Head and neck (squamous)	Skin (moderate), anorexia (moderate)	<50% regression: then sudden death from carotid artery hemorrhage
20	Tonsil (squamous)	Pulmonary (?) vs aspiration pneumonia	Mixed: almost complete regression of nasopharyngeal mass, progressive invasion of skull
21	Nasopharynx (squamous)	Pulmonary (?), total dose exceeded 400 mg	PD
22	Head and neck (squamous)	Anorexia, nausea and vomiting (severe)	PR
23	Head and neck (squamous)	Skin (mild)	PR: transient but dramatic, progressed on continued treatment
24	Head and neck (squamous)	Neuropathy (severe)	<50% decrease in pleural neoplastic effusion
25	Lymphoepithelioma	Skin (mild)	PR
26	Head and neck (squamous)	Skin (mild)	PD: ineligible because of extensive prior bleomycin therapy
27	Mesothelioma	Skin (mild)	No evaluable disease
28	Hodgkin's disease	Skin (mild)	<50% (nodes decreased)
29	Hodgkin's disease	Skin (mild)	PR: also received NSC-45388‡ (inevaluable)
30	Undifferentiated lymphoma	Skin (mild)	NR
31	Testicular	None	PR: disappearance of 5 × 5-cm epigastric mass
32	Melanoma	Skin (moderate), anorexia (moderate), neuropathy (moderate)	NR
33	Renal cell	Skin (moderate)	PD
34	Carcinoma (anus)	Skin (moderate)	PR: CR of perineal mass, no change in pulmonary nodules
35	Carcinoma (breast)	None	PD
36	Carcinoma (esophagus)	Skin (severe), received radiotherapy at same time	NR

*NR = no response; PR = partial response; PD = progressive disease.
†5-FU: NSC-19893; CAS reg. No. 51-21-8; 5-fluorouracil.
‡NSC-45388: CAS reg. No. 4342-03-4; 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; DIC.

studied, four of 15 evaluable patients (27%) had a partial response. These included one of five patients with squamous cell carcinoma, two of nine patients with adenocarcinoma, and one of one patient with oat cell carcinoma. Three of nine evaluable patients with head and neck cancer, including one patient with a lymphoepithelioma, had a partial response. Responses were seen in additional single patients with testicular cancer (mixed cell type) and cloacogenic carcinoma, for a total of nine among 32 patients evaluated (28%). As in other studies with bleomycin alone, the duration of response was short. Most patients showed evidence of disease progression within a few weeks after therapy was discontinued, in spite of an attempted maintenance with other drugs.

Toxicity

Mild skin toxic reactions and neuropathy were seen in almost all patients. Severe vincristine neuropathy developed in only one patient in spite of the rather high total dose of this agent administered over a short period of time. Only one patient had any indication of a myelosuppressive toxic manifestation, and this occurred in patient No. 13 who had isolated thrombocytopenia which may have been associated with unsuspected marrow involvement by tumor.

One of the 36 patients (patient No. 8) may have died of bleomycin-induced pulmonary damage, but death occurred in another hospital without an autopsy. Another patient (patient No. 7) who died 2 months later from progressive tumor and cardiac disease, had an episode of acute respiratory failure associated with a diffuse, bilateral infiltrate which was shown on chest roentgenogram. The respiratory failure did not respond to broad-spectrum antibiotics but was dramatically resolved with the addition of high-dose steroid therapy. This clinical course appears to be characteristic of reversible but severe bleomycin toxicity (12). In an additional four patients, bleomycin pulmonary toxicity was suspected, but in none was it of clinical significance or documented by autopsy findings. In all of these patients, the drug was immediately discontinued when toxicity was noted. Therefore, probable, clinically important bleomycin toxicity occurred in two of 36 patients (6%), and possible, subclinical or suspected toxicity occurred in six of 36 patients (17%).

These figures are in accord with the incidence of this complication as reported by others (2)[7] who used bleomycin alone. No potentiation of pulmonary fibrosis, or of the other known toxic effects of the two agents, were observed, and no unexpected side effects were seen.

DISCUSSION

Our overall response rate for lung cancer in this kinetically based, sequential study of vincristine and bleomycin was 27%. This compares favorably with the previous response rate of 5% for bleomycin alone, in an evaluation by the Southwest Cancer Chemotherapy Study Group. Although the comparison was not controlled, the bleomycin was given in essentially the same way. Therefore, we feel it is likely that the combination of vincristine and bleomycin, given in the dose and schedule used in this study, is superior to bleomycin alone for the treatment of lung cancer, and further trials of the combination are warranted to test the validity of this conclusion.

Also indicated for the future are kinetic studies on patients receiving this combination to determine how the drugs affect the tumor growth fraction and the cell cycle at various points in time. Preliminary work in this direction is under way in our department.

The greatest potential of this combination may be further combination with other agents which have similar antitumor efficacy without similar toxicity, such as the myelosuppressive antimetabolites or alkylating agents. Attempts to exploit possible synergism of such combinations are currently under investigation.

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Clinical Evaluation of 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU; NSC-409962) With Fluoxymesterone (NSC-12165) in the Treatment of Solid Tumors [1,2,3]

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SUMMARY

BCNU, a drug with alkylating properties, was given to 60 patients with solid tumors. Because of the severe hematopoietic toxic effects of the drug and the reported myeloprotective action and erythropoietic stimulation of androgens, these 60 patients were randomly selected for either of two treatment programs: one group received BCNU alone and the other group received BCNU plus the androgen fluoxymesterone. Six of 47 evaluable patients had partial remissions during this study: two who received BCNU alone and four who received BCNU plus fluoxymesterone. Fifty-six patients were evaluable for toxicity; peak hematologic toxicity was delayed and prolonged. Anemia was not a problem and was present only when patients were bleeding in association with severe thrombocytopenia. The nadir of leukopenia was reached between the fifth and sixth week; recovery usually occurred by the seventh week. Thrombocytopenia was constant and severe; the nadir occurred in the fourth week and recovery occurred by the sixth week. Toxic effects that developed during the two treatment programs were basically the same and were not altered by the use of fluoxymesterone.

[Cancer Chemother Rep 57:225–230, 1973]

BCNU, from the group of nitrosoureas, has been reported to have promising results in the treatment of Hodgkin's disease and meningeal leukemia (1,2). Ahmann found BCNU ineffective in the treatment of metastatic breast cancer (3). Subsequent to this, there were several clinical trials of the drug in a wide spectrum of tumors. From previous clinical studies, a constant and unique feature of BCNU was the severe hematologic toxic effects which were delayed in onset and of a more prolonged duration than those which had been observed in other drugs. However, we felt that a more

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[3] BCNU: CAS reg. No. 154-93-8.

Fluoxymesterone: CAS reg. No. 76-43-7; androst-4-en-3-one, 9-fluoro-11β,17β-dihydroxy-17-methyl-.

Both drugs were supplied by the Cancer Therapy Evaluation Branch, Division of Cancer Treatment, NCI.

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extensive evaluation of BCNU in the treatment of human solid tumors was indicated as a result of these early reports.

Since androgens have a possible myeloprotective action and are also known to stimulate erythropoiesis (4–7), the present clinical study was designed to determine if the hematopoietic toxicity of BCNU could be modified if the drug was used in combination with androgens.

MATERIALS AND METHODS

BCNU was supplied as a lyophilized powder in 100-mg vials; 3 ml of absolute ethanol and 27 ml of sterile normal saline were added. This drug preparation was added to 100–125 ml of normal saline and was given by intravenous (iv) infusion over a 10–15-minute period. After the infusion, the vein was flushed with a 5% dextrose solution to prevent local irritation. Fluoxymesterone was supplied in compressed 10-mg tablets.

Patients were selected according to the following criteria: histologic evidence of malignancy; measurable lesion; an expected survival of at least 2 months; an adequate marrow reserve which included a white blood cell count (wbc) greater than 4000 cells/mm^3, a platelet count greater than 100,000 platelets/mm^3, and a hemoglobin level greater than 10 g/100 ml; no prior chemotherapy for 3 weeks; and evidence of complete bone marrow recovery.

Pretreatment Laboratory Studies

The following studies were required for each patient: complete blood cell count and platelet count; chest roentgenogram and metastatic bone survey; and determinations of blood urea nitrogen (BUN) level, creatinine clearance, alkaline phosphatase level, serum glutamic oxaloacetic transaminase (SGOT) level, and bilirubin level.

Evaluation During Study

Physical examinations of the patients and measurements of tumor masses were performed at 6-week intervals. Hematopoietic indices were repeated at weekly intervals. All other laboratory studies and roentgenograms were repeated at 4-week intervals.

Dose Schedules and Administration

Patients were given BCNU alone or in combination with 30 mg/day of fluoxymesterone during the entire period of the study. Initially, patients who were considered to be good risks were given BCNU at a dose level of 100 mg/m^2/day for 3 days, and patients who were considered poor risks were given 75 mg/m^2/day for 3 days as induction therapy. However, because of the severe hematopoietic toxicity observed in the first 25 patients (13 patients, BCNU alone; 12 patients, BCNU and fluoxymesterone), the induction regimen was changed to one daily dose for 2 days at the same dose level as before. Six weeks after induction therapy, all of the patients were maintained on 50 mg/m^2 given iv once a week if they had recovered from the initial hematopoietic toxic effects and their diseases had not progressed.

Patient Selection and Classification

Patients with a history of previous chemotherapy and/or radiation to the bone at a dose of 3000 rads or more to an area of 15 × 15 cm or greater were automatically placed in the category of poor risk patients. A total of 60 patients with a variety of malignant tumors were randomly selected for either of the two treatment programs. Thirty-three patients received BCNU alone and 27 patients received BCNU plus fluoxymesterone.

Definition of Response

Complete response—disappearance of all clinical evidence of the disease; *partial response*—decrease in the product of the longest and widest diameter by 50% or more of all measurable tumor masses; *no change*—increase or decrease in the product of the two diameters by a factor of less than 50%; *progression*—increase in the product of the two diameters of the measured lesions by a factor of 50% or more, or the development of new lesions.

RESULTS

A total of 60 patients were entered into the study and were almost equally divided between the two types of treatment programs. The number of patients and the types of tumors

included in the study were: breast (16 patients), gastrointestinal (14), malignant melanoma (5), bladder (3), pancreas (3), head and neck (3), prostate (2), and ovarian (2). There were ten unknown primary tumors, and one each of endometrial, osteogenic sarcoma, cervical, hepatic, bile duct, and alveolar cell tumors.

Sixty patients were entered into the study; 47 were evaluable. Thirteen patients were considered unevaluable for the following reasons: protocol violation (3), early death (7), inadequate data (1), inadequate treatment (1), or lost to followup (1). Of the 47 evaluable patients, six patients (13%) had partial responses, 12 had no change in their status, and 28 had progression of their tumors. If the seven patients who were classified as unevaluable due to early death were included in the group of patients considered evaluable, the response rate was 11%. Twenty-four of the evaluable patients were given BCNU alone and 23 received a combination of BCNU and fluoxymesterone.

Six patients had partial responses: four patients with breast cancer, one with rectal carcinoma, and one with an epidermoid carcinoma of unknown origin. Five of these patients had disseminated systemic metastases and one patient's disease was an extensive local recurrence of breast carcinoma to the skin of the chest wall. There were four patients treated with the combination of BCNU and fluoxymesterone and two patients were treated with BCNU alone. The possibility of androgen influence on the response in breast tumors cannot be ruled out; all patients but one had had prior androgenic hormonal therapy and were no longer responsive to such therapy. The median duration of response in these six patients was between 4 and 6 weeks; the longest response lasted 52 weeks. None of the patients who failed to obtain regressions with induction therapy had regressions during the maintenance regimen.

TOXIC EFFECTS

Hematologic Toxicity

Fifty-six patients were evaluable for toxicity. Patients were considered evaluable for toxicity if they had had hematologic studies at least every 2 weeks for a period of 6 weeks. Patients who died before 6 weeks, in whom death could possibly be attributed to the therapy, or who had not recovered from toxic effects at the time of death were considered evaluable for toxicity. For evaluation of toxicity, all patients, both good risk and poor risk, were combined. The numbers of patients in the poor risk category for both the 2-day and the 3-day regimens were small and the observed toxic effects were almost equal for both groups. Only four patients were not evaluable for toxicity studies: two patients died within 2 weeks of the start of therapy, one patient was lost to followup but was alive at 6 weeks, and one patient received only 1 day of therapy.

The average response of peripheral wbc and platelet counts is shown for each of the four treatment regimens in figures 1–4. Since one of the major purposes of this study was to determine whether fluoxymesterone could protect patients from the hematopoietic toxic effects of BCNU, each chart compares the group of patients given BCNU alone with the group of patients given BCNU plus fluoxymesterone for each evaluated treatment regimen. The 3-day regimen produced significantly greater leukopenia and thrombocytopenia (figs 1 and 2). The nadir of leukopenia for both groups was reached between the fifth and sixth week. The average lowest wbc was 2400 cells/mm^3 for the group given BCNU alone and 3100 cells/mm^3 for the group given BCNU plus fluoxymesterone. Likewise, platelet counts decreased approximately the same; the nadir occurred at 4 weeks for both groups and recovery occurred at 6 weeks. The average lowest platelet count was 66×10^3 for the group treated with BCNU alone and 77×10^3 for the group treated with BCNU plus fluoxymesterone. Neither of these effects were considered statistically different ($P>0.10$).

Sixteen patients received the 2-day regimen of BCNU alone and 15 patients received BCNU for 2 days plus daily fluoxymesterone. Figures 3 and 4 compare the effects of the treatment on the wbc and platelet counts of patients who received only the 2-day induction course. Again, the pattern of depression and recovery for

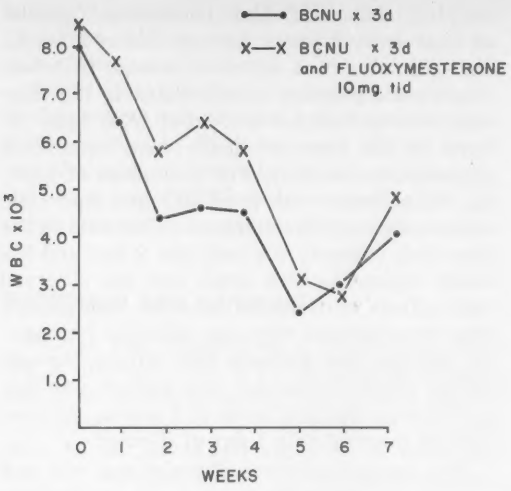

FIGURE 1.—Comparison of effects of BCNU alone and BCNU plus fluoxymesterone on wbc when given for 3 days.

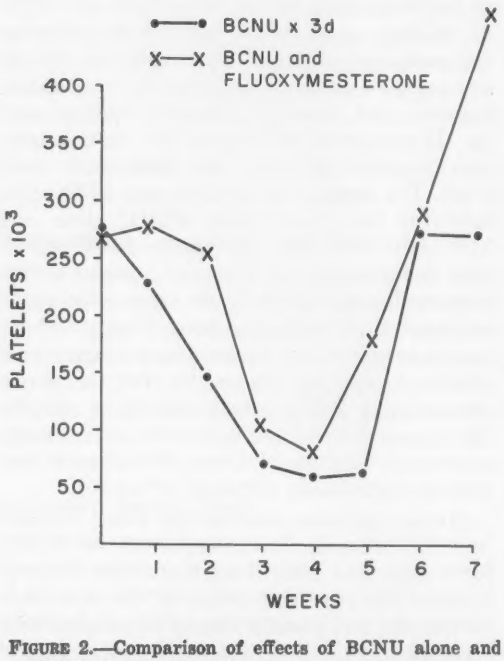

FIGURE 2.—Comparison of effects of BCNU alone and BCNU plus fluoxymesterone on platelets when given for 3 days.

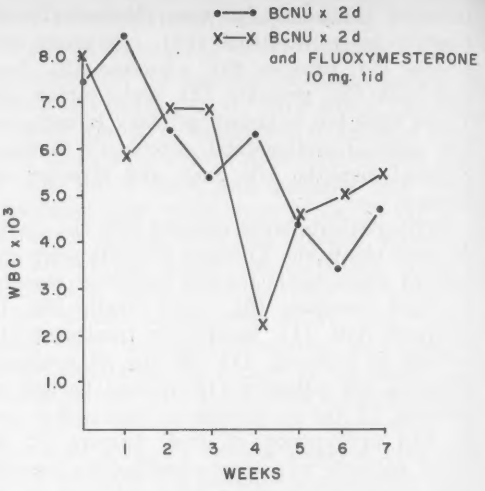

FIGURE 3.—Comparison of effects of BCNU alone and BCNU plus fluoxymesterone on wbc when given for 2 days.

both groups was similar. The nadir of leukopenia occurred at 6 weeks for the group given BCNU alone and at 4 weeks for the group given BCNU plus fluoxymesterone; the average lowest wbc counts were 3400 and 2200 cells/mm³, respectively. Platelet counts were also diminished less significantly in this 2-day treatment program than in the 3-day regimen, and again there was no difference between the BCNU alone and BCNU plus fluoxymesterone groups (fig 4). Hemoglobin level depression also was evaluated in the four groups; there was no depression at 6 weeks in any of the four therapeutic regimens.

Gastrointestinal Toxicity

Nausea and vomiting occurred in only nine patients; stomatitis occurred in two patients. Some patients complained of having dry mouths, and a metallic taste occurred in one patient while the drug was being given iv.

Other Side Effects

A throbbing headache and a hot flush while the drug was being administered were included as other side effects. At first, it was thought

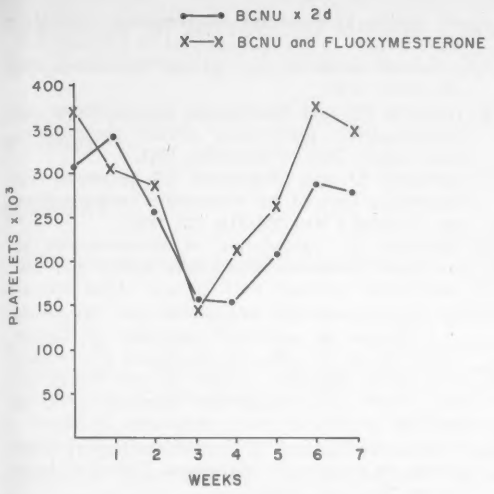

FIGURE 4.—Comparison of effects of BCNU alone and BCNU plus fluoxymesterone on platelets when given for 2 days.

that these reactions were due to the use of ethanol, but when ethanol was given separately these effects were not reproduced. Hypoglycemia was also ruled out by taking a normal blood sugar specimen while the patient was having these symptoms.

Acute pulmonary edema developed in one patient; this reaction had been noted by previous investigators. The exact mechanism of action for this side effect is unknown.

Phlebitis and pain at the injection site resulted if the drug extravasated into the tissues. For this reason, the vein was flushed with 5% dextrose in water after the drug was given. Confusion was noted in three patients and was usually accompanied by hematologic toxic effects. Alopecia occurred in only one patient. A gastric ulcer developed in one patient who died of hemorrhage from the ulcer.

DISCUSSION

BCNU has demonstrated activity against transplanted rodent tumors such as Wagner osteogenic sarcoma, L1210 leukemia, sarcoma 180, Ehrlich ascites carcinoma, Jensen sarcoma, Iglesias ovarian tumor, Lewis bladder carcinoma, Walker carcinoma 256, Friend virus leukemia, and glioma 26 (1,8). Clinically good success has been reported with central nervous system (CNS) tumors, leukemia, Hodgkin's disease, malignant melanoma, carcinoma of the esophagus, bronchogenic carcinoma, Ewing's sarcoma, carcinoma of the cervix, and mycosis fungoides (2,9,10,12). Even though the mechanism of action of BCNU is not clear, its major mechanism of action is believed to be that of an alkylating agent (1). In early clinical trials with BCNU, two unique features were observed: (*a*) its ability to cross the blood-brain barrier (13), thus making it suitable for the treatment of CNS tumors and meningeal leukemia; and (*b*) the delayed and unexplained onset of hematologic toxicity which occurs 3–5 weeks after the administration of the drug. This toxicity frequently is prolonged with complete recovery occasionally not attained for up to 8–10 weeks after therapy; recovery is usually complete within 6–7 weeks.

The Eastern Cooperative Oncology Group reported their experience with BCNU in the therapy of 144 patients. Good results were noted in some advanced neoplasms. They found 125 mg/m² for 3 successive days to be a tolerable dose level (10).

Reitemeier reported nominal responses in patients with advanced gastrointestinal cancer (14). Lessner, who had good to excellent responses in 15 of 30 patients with advanced Hodgkin's disease, used a loading dose of 100 mg/m² for 2 successive days followed 4 weeks later with a maintenance dose of 100 mg/m² every 2 weeks as tolerated (2). Iriarte et al obtained remissions of CNS manifestations in three of six patients with CNS leukemia (11).

Severe hematopoietic toxicity has been the primary limiting factor of BCNU; androgens have been suggested as protective factors when used in combination with cytotoxic agents. Therefore, a randomized study was designed using BCNU alone and BCNU in combination with fluoxymesterone to see if this anabolic androgen would minimize the degree or duration or both of hematopoietic toxicity.

Brodsky and Kahn initially reported that testosterone enanthate [8] was more protective in

[8] Testosterone enanthate: NSC-17591; CAS reg. No. 315-37-7; androst-4-en-3-one, 17β-hydroxy-, heptanoate.

patients with hematologic cancer than in patients with solid tumors (15). In the present study, 56 patients were evaluable for toxicity. Serial hemoglobin levels, wbc and platelet count determinations, degree of toxicity, and eventual recovery were essentially the same for patients who received BCNU alone as for those who received BCNU plus fluoxymesterone. Patients who were treated on the 3-day regimen had lower values of wbc and platelet counts than those treated on the 2-day schedule. This report is at variance with the reports of Brodsky et al (7,15), but it should be pointed out that the differences might be attributed to variations in experimental design. For example, a different anabolic androgen was used (fluoxymesterone was used in the current study versus testosterone enanthate or nandrolone phenylpropionate [9] in the earlier study), and each patient served as his own control in the Brodsky et al series. A possible explanation for the failure of androgens to modify the toxicity of BCNU is that perhaps these patients were not given androgen therapy before the introduction of BCNU. It has been suggested that the marrow-stimulating effects of androgens are not evident for 4-6 weeks (5,6). However, this is the period during which BCNU had its peak marrow-suppressant effect.

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[9] Nandrolone phenylpropionate: NSC-23162; CAS reg. No. 62-90-8; estr-4-en-3-one, 17β-hydroxy-, hydrocinnamate.

Response of Advanced Breast Cancer to Two Dosage Regimens of 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU; NSC-409962)[1,2]

John D. Bonnet, Robert W. Brownlee, V. K. Vaitkevicius, and Robert W. Talley [3,4]

SUMMARY

Ninety-one patients with far-advanced breast cancer were treated with one of two dosage schedules of BCNU: regimen I, 100 mg/m² for 2 days every 6 weeks; or regimen II, 50 mg/m² weekly for 6 weeks. Of the 66 evaluable patients, 13 of 36 responded to regimen I and six of 30 responded to regimen II. A comparison of the results failed to show a significant difference in toxicity, response rate, duration of response, or survival. Mean response duration and survival for both groups was 6 months or less. The best responses were seen in patients with skin metastases (11 of 21 patients).

[Cancer Chemother Rep 57:231–234, 1973]

The initial report demonstrating the antitumor activity of BCNU did not evaluate the response in patients with breast cancer (1). In a subsequent preliminary trial of BCNU, four of 13 patients with widely disseminated breast cancer had objective remissions about 6 weeks after drug administration (2). The initial dose schedule was 100 mg/m² on each of 3 consecutive days but it was changed to 100 mg/m² for 2 days when severe and delayed hematopoietic toxicity was observed.

The protocol for this study was designed to establish the response rate of BCNU in patients with breast cancer. Two dosage regimens were evaluated to determine if there was an optimal schedule for this agent in the treatment of patients with far-advanced disease: regimen I, 2 days of BCNU treatment every 6 weeks; regimen II, weekly administration of BCNU. After reviewing a recent report stating that patients with end-stage disease did not respond to this treatment (3), the study was extended to correlate the duration of survival with the response.

MATERIALS AND METHODS

The 91 patients with far-advanced breast cancer entered in this study had failed to respond either initially or after a temporary response to hormonal therapy. Five patients had not received previous cytotoxic therapy. Seven of nine patients who had received only

[1] Received Feb 1, 1973; revised Apr 13, 1973.

[2] BCNU: CAS reg. No. 154-93-8. Supplied by the Cancer Therapy Evaluation Branch, Division of Cancer Treatment, National Cancer Institute (NCI).

[3] This study (Jan 1970–June 1971) was done by The Southwest Cancer Chemotherapy Study Group (grant CA-03754). The following institutions participated in the study: Scott and White Clinic, Temple, Tex (grant CA-10187, Dr. Bonnet); Henry Ford Hospital, Detroit, Mich (grant CA-04915, Drs. Brownlee and Talley); Wayne State University, Detroit, Mich (Dr. Vaitkevicius); University of Arkansas, Little Rock (grant CA-03400); University of Texas Medical Branch, Galveston (grant CA-03096); Louisiana State University, New Orleans; M. D. Anderson Hospital, Houston, Tex (grants CA-10376 and CA-10379); Ohio State University, Columbus (grant CA-04920); University of New Mexico, Albuquerque (grant CA-12213); University of Kansas, Kansas City (grant CA-12644); University of Virginia, Charlottesville; Keesler Air Force Base, Miss; and Brooke Army Hospital, San Antonio, Tex. All grants are from the NCI, National Institutes of Health, Department of Health, Education and Welfare.

[4] *Reprint requests to:* The Southwest Cancer Chemotherapy Study Group, Operations Office, 3500 Rainbow Blvd, Suite 201, Kansas City, Kan 66103.

one drug had been treated with 5-fluorouracil (NSC-19893; CAS reg. No. 51-21-8). More than one cytotoxic drug had been administered to the remainder of the patients and more than 50% of them had received radiotherapy to at least one site. Before therapy, all patients had a white blood cell (wbc) count greater than 4000 cells/mm^3 and a platelet count greater than 100,000 cells/mm^3. During the study, complete blood cell and platelet counts were done at weekly intervals. Hepatic and renal states were determined initially and at least every 6 weeks by biochemical tests. Radiologic surveys of bones (including, as a minimum, surveys of the lateral skull, lateral lumbar spine, and anteroposterior pelvis and hips) were obtained initially and at periods of at least every 12 weeks.

Patients were randomly allocated by a statistically designed system to receive one of two dosage schedules of BCNU. Forty-eight patients received regimen I, 100 mg/m^2 of BCNU on each of 2 successive days every 6 weeks. Thirty-one of the remaining 43 patients received regimen II, 50 mg/m^2 of BCNU administered weekly for 6 weeks. Initially, 12 patients receiving regimen II were given 30 mg/m^2 weekly for 6 weeks. However, when the lower weekly dosage failed to produce adequate response or significant toxicity, the weekly amount was increased to 50 mg/m^2. To be considered evaluable, the following criteria were required: regimen I—the patient must receive two treatments at a total dose of 400 mg/m^2 and survive for 6 weeks after the last treatment; regimen II—the patient must receive six weekly treatments at a total dose of 300 mg/m^2 and survive for 6 weeks after the last treatment.

A partial response was indicated when the sum of the products of the diameters of all tumor masses decreased in size by 50% and the patient was without apparent new disease for at least 6 weeks. None of the patients had static disease or a complete response.

Gastrointestinal toxicity was indicated as mild, moderate, or severe, and was evaluated by the investigator according to the difficulty he had in controlling it. Hematologic toxicity was rated as mild, moderate, or severe. Mild toxicity included wbc counts as low as 2500 cells/mm^3 and/or platelet counts between 75,000 and 100,000 cells/mm^3. Severe toxicity was defined as a wbc count of less than 1500 cells/mm^3 and/or a platelet count of less than 40,000 cells/mm^3. The range of wbc and/or platelets between these two limits was defined as moderate hematologic toxicity.

RESULTS

Responses to BCNU therapy were evaluable in 66 of the 91 patients. Sixteen patients were lost to the series because of early death. Nine other patients were excluded: seven because of insufficient data and two for protocol violations. Of the 36 evaluable patients receiving regimen I, 13 (36.1%) responded. Six of 30 evaluable patients (20.0%) receiving regimen II responded (fig 1). The difference in the response rates for the two groups of patients was not statistically significant. The overall response rate was 28.7% (19 of 66 patients).

Responses occurred within the first 3 months except for one patient treated with regimen II who responded in 5 months. For patients who responded, the total dosage of BCNU and the duration of response were not significantly different regardless of whether they received regimen I or regimen II. The mean duration of response was about 3 months for all patients.

All of the patients died of progressive metastatic disease. Survival curves were analyzed (4, 5), and the differences in survival between responders and nonresponders and between regimen I and regimen II patients were not significant. The 50th percentile for mean survival for responders was 6 months for patients receiving either regimen I or regimen II; for nonresponders, it was 4.5 and 5 months respectively. The longest period of survival was 18 months.

Toxicity is correlated with the response to the two dosage schedules of BCNU in table 1. Although regimen I may have seemed to be more toxic and to have produced a greater response, the differences between the two regimens were not significant. Previous chemotherapy increased toxicity, but the adverse effects were not significantly different in the two groups. Four of the 14 patients (35%) with minimal previous therapy responded.

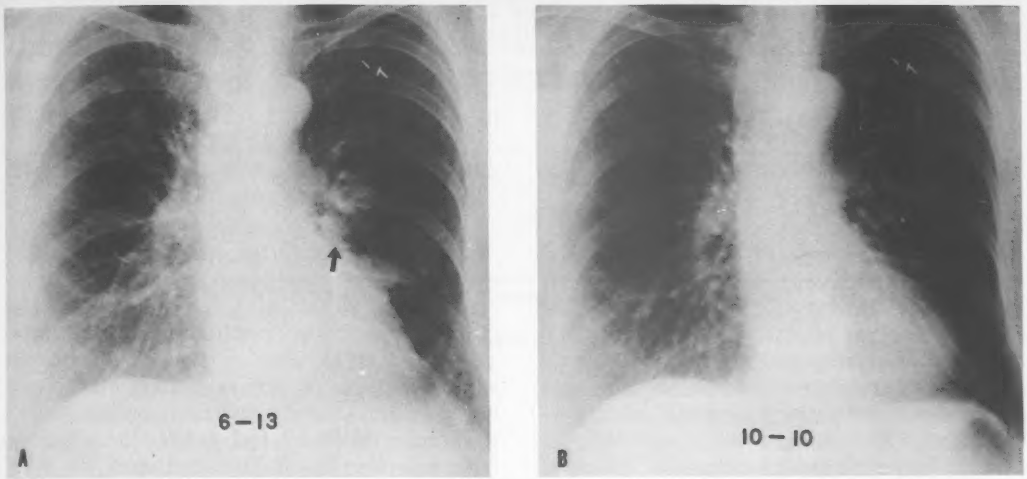

FIGURE 1.—Chest roentgenograms of a 70-year-old woman who had a partial response to regimen II in August 1970. Improvement continued until October. She had generalized metastases when she died on November 18, 1970. (*A*) Before therapy. (*B*) Improvement seen after therapy.

TABLE 1.—Correlation of response to BCNU therapy with toxicity in 66 evaluable patients

	Regimen I			Regimen II		
Toxicity	No. of evaluable patients	Partial responses	No response	No. of evaluable patients	Partial responses	No response
None	10	1	9	8	2	6
Mild	9	4	5	11	1	10
Moderate	7	3	4	6	2	4
Severe	8	3	5	5	1	4
Total	36	13	23	30	6	24
Total with toxicity (%)	26(72.2)	12(92.3)	14(60.8)	22(73.3)	4(66.7)	18(75.0)

TABLE 2.—Correlation of responses to BCNU therapy with menopausal status (72 patients*)

Classification of patients	Years postmenopausal	
	0–5(%)	>5(%)
Responders	3(9.3)	13(32.5)
Nonresponders	22(68.7)	18(45.0)
Early death	7(22.0)	9(22.5)
Total	32	40

*Insufficient data for evaluation of 19 patients.

Patients who were 5 years or more postmenopausal responded better than those who were less than 5 years postmenopausal (table 2). The site of the lesion influenced the response rate more than any other factor (table 3). Eleven of 21 patients (52.3%) with at least skin metastases responded. Eight of these patients received regimen I. Of the 28 patients with pulmonary metastases, eight showed evidence of lymphatic spread, but only one (12.5%) responded. Although a theoretical advantage of BCNU may be its ability to cross the blood-brain barrier and control metastases, none of three patients with central nervous system involvement responded.

COMMENT

In contrast to earlier studies in which none

TABLE 3.—Correlation of response to BCNU therapy with sites of metastatic lesions (66 evaluable patients)

Site of lesion	No. of sites: Evaluable patients* (66 total)	No. of sites: Responders† (19 total)	Percentage
Skin	21	11	52.3
Lung	28	6	21.4
Bone	31	4	12.9
Liver	16	2	12.5

*Twenty-four patients had disease in 2 sites; 3 patients had disease in 3 sites.

†Four patients had disease in 2 sites.

of 16 patients responded to BCNU alone (3) and none of six patients responded to BCNU combined with cytosine arabinoside (6),[5] 19 of our 66 patients with advanced metastatic breast cancer responded to BCNU therapy for a short period of time. We agree with Ahmann (7), that the possible clinical variables affecting the response rate (ie, age, sex, extent of disease, cell type, grade of malignancy, free interval, menstrual status, and previous treatment) should be considered in a drug analysis. When we did this, a high incidence of patients with responsive skin lesions was noted; however, this factor failed to reveal why 19 of our 66 patients responded to BCNU therapy and why none of the 16 patients reported by Ahmann and his colleagues (3) responded. When survival curves were plotted and the difference in slope was computed for our series, the survival curve of responders versus nonresponders was slightly but not statistically different. We can only conclude that some patients with breast cancer do show a partial short-term response to BCNU therapy that apparently does not prolong survival.

No clearcut advantage of our regimen I over regimen II can be claimed, except that receiving two intravenous injections on consecutive days may be more convenient for a patient than receiving six weekly injections.

[5] Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

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Urinary Excretion of β-Glucuronidase After Administration of Streptozotocin (NSC-85998) to Fischer Rats [1,2,3,4]

Brief Reports and Preliminary Communications

Gaylord M. Conzelman, Jr., and David H. Gribble [5]

Streptozotocin, an antibiotic from *Streptomyces acromogenes*, has been used in the treatment of islet cell carcinoma in man (1–3). Although streptozotocin produces insulin-dependent diabetes in rats (4), Sadoff (5) found that man is relatively resistant to the diabetogenic action of the drug. Although renal toxic effects in rats injected with streptozotocin have not been reported, Sadoff (6) described renal tubular toxic manifestations in all 18 cancer patients treated with this drug in his study. The nephrotoxicity ranged from an isolated defect to a full-blown Fanconi's syndrome.

Previous unpublished studies in our laboratory have shown that the urine specimens collected from rats treated with neomycin, a nephrotoxic antibiotic, had greater β-glucuronidase activities than 24-hour urine samples collected from untreated control rats. The experiment described in this current report was a preliminary attempt to evaluate the usefulness of urinary β-glucuronidase assay as a method for detecting nephrotoxic effects in rats injected with single doses of streptozotocin.

MATERIALS AND METHODS

Streptozotocin dissolved in citrate buffer (0.005 M, pH 4.6) was injected intraperitoneally (ip) into female Fischer rats weighing 90–100 g (obtained from Simonsen's Laboratory, Inc., Gilroy, Calif). The animals were divided into three groups: animals in group *B* were given 50 mg/kg of streptozotocin, those in group *C* were given 100 mg/kg of streptozotocin and those in group *A* were injected with citrate buffer only. As suggested by Coonrod and Paterson (7), the preputial glands were surgically removed from all rats 1 week before the beginning of the experiments. Ether was used for anesthesia.

Twenty-four-hour urine specimens from rats housed individually in stainless steel metabolism cages were collected into iced vessels and were assayed for β-glucuronidase activity according to a method previously described (8). The method used was essentially that of Talalay et al (9), modified so that the substrate, phenolphthalein-mono-β-glucuronic acid (Sigma Chemical Co., St. Louis, Mo), was 0.2 mM and the assay was carried out at a pH value of 5.2. Two-tenths ml of urine (diluted 1:5 with distilled water and sterilized by passage through a filter[6]), 0.2 ml of acetate buffer (0.2 M, pH 5.2), and 0.2 ml of substrate (6 mM phenolphthalein-mono-β-glucuronic acid solution adjusted to pH 5.2) were incubated in stoppered test tubes for 15 hours at 38 C. A blank tube containing no urine was also incubated. After removal from the water bath, 0.2 ml of urine was added to the blank and 0.2 ml of 10% sodium carbonate solution and 1 ml of glycine buffer (0.2 M, pH 10.6) were added to each tube. The colored solutions were read against the blank in a Klett colorimeter fitted with a No. 54 filter. The enzyme activity was expressed in units, 1 unit being the quantity of enzyme which would liberate 1 μg of phenolphthalein/hour of incubation. The test of signi-

[1] Received Aug 9, 1972; revised Mar 26, 1973; accepted Apr 5, 1973.

[2] Reported in part at the 5th International Congress on Pharmacology, San Francisco, Calif, July 28, 1972.

[3] Supported in part by General Research support grant FR-05457.

[4] Streptozotocin was supplied by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute.

[5] Departments of Physiological Sciences and Veterinary Pathology, School of Veterinary Medicine, University of California at Davis.

[6] Millipore HA in a Swinny Hypodermic Adapter.

ficance was calculated using the "Student's t" test; the level of significance was chosen as $P < 0.05$ (one-tailed).

On the 5th day after treatment the animals were killed, and their kidneys were removed and fixed in neutral formalin. The fixed tissues were later sectioned and stained with hematoxylin and eosin.

RESULTS AND DISCUSSION

The results shown in table 1 indicate that urine collected from rats injected ip with single doses of streptozotocin showed higher β-glucuronidase activity than urine from control rats. The data also indicate that the β-glucuronidase activity was greater in 24-hour urine specimens collected from rats that received the higher dose.

Stained sections of kidneys from two of the 12 animals who received the larger dose of streptozotocin showed focal tubular necrosis. In the other ten animals, cytoplasmic vacuolization was greater than normal. The lesions, in their severest form, were mild.

The results presented in this report suggest that the increase in urinary β-glucuronidase activity was the result of renal tubular damage and that doses of streptozotocin which produced only minimal morphologic lesions effected an appreciable increase in the output of urinary β-glucuronidase. Preliminary observations made by the use of acrylamide gel electrophoresis suggest that the increased β-glucuronidase activity was due to an increased enzyme concentration rather than to decreased β-glucuronidase inhibitors or increased β-glucuronidase activators.

The authors concur with Coonrod and Paterson (7) who concluded that the assay of urinary β-glucuronidase is a simple, inexpensive, and reproducible test which permits evaluation of β-glucuronidase excretion in the rat as an expression of renal injury. Further studies may show that β-glucuronidase and certain other urinary enzymes can be used as part of the preclinical testing of new drugs in an effort to detect potential nephrotoxic agents.

TABLE 1.—β-Glucuronidase activities in the urines of untreated rats and rats treated with streptozotocin*

Group	No. of rats	Streptozotocin (mg/kg)	Urinary β-glucuronidase (units/24-hr urine sample) Day 1	Day 2	Day 3	Day 4	Day 5
A	12	0	59±10	43±6	50±7	59±6	60±8
B	12	50	66±10	62±13	76±11†	109±16†	139±30†
C	12	100	86±21	165±85	127±57	114±18†	184±40†

*The data represent the means (± SE) of assays carried out on 24-hr urine specimens collected from individual rats.

†$P < 0.05$ (one-tailed).

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Multiple-Drug Therapy for Malignant Solid Tumors in Adults [1]

Robert E. Gerner and George E. Moore [2]

Brief Reports and Preliminary Communications

A number of encouraging studies using combination chemotherapy for the systemic treatment of solid tumors have been reported (1–3). This report summarizes the followup of 48 patients with various types of solid tumors treated with a combination of 5-fluorouracil (5-FU), cyclophosphamide, methotrexate, and vincristine.[3] This treatment was compatible with outpatient care and had no major risk of debilitating toxicity.

PATIENTS AND METHODS

All patients had a histologic diagnosis of malignancy with measurable lesions and had received no prior radiation therapy or chemotherapy for a period of 2 months. The various histologic types and numbers of evaluable patients in each group of this mixed tumor study are listed in table 1. The following multiple-drug protocol was employed. The initial loading course consisted of 5-FU, 10 mg/kg given intravenously (iv) on Days 1–4; vincristine, 0.025 mg/kg given iv on Day 1; cyclophosphamide, 100 mg given orally on Days 2–4; and methotrexate, 10 mg given orally on Days 2–4. Maintenance therapy consisted of 5-FU, 300–500 mg given iv once weekly; vincristine, half-loading dose given iv once weekly; cyclophosphamide, 100 mg given orally three times weekly; and methotrexate, 2.5–5 mg given orally three times weekly. Adjustments in dosage were made in relation to toxicity. A period of 2 or more months was considered an adequate trial of treatment.

[1] Received Mar 20, 1973; accepted Mar 23, 1973.

[2] Public Health Research Group and Department of Surgery, Roswell Park Memorial Institute, New York State Department of Health, Buffalo.

[3] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

TABLE 1.—Tumor type and response

Tumor type	No. of patients treated	No. of patients with—			
		Objective response*	Lesser response	Stationary disease	Progression
Adenocarcinoma, colon	14	2(14%)	4	5	3
Adenocarcinoma, stomach	3	1(33%)			2
Osteogenic sarcoma	5	1(25%)	2	1	1
Carcinoma, lung	5	0	1		4
Soft tissue sarcomas (rhabdomyosarcoma, liposarcoma, fibrosarcoma, and leiomyosarcoma) and synovial sarcoma	10	0	1		9
Squamous cell carcinoma, neck	2	0	2		
Carcinoma, pancreas	1	0			1
Adrenal carcinoma, histiocytoma, and mesothelioma	3	0			3

*Combination of complete and partial response.

TOXICITY

Toxic manifestations are summarized in table 2. Leukopenia (white blood cell count of 800 cells/mm³) and thrombocytopenia (platelet count of 30,000 cells/mm³) occurred in two patients requiring hospitalization and supportive care. All other toxic manifestations were reversible by temporarily withholding therapy and the patients did not require hospitalization.

TABLE 2.—Toxicity

Toxic effect	No. of patients with toxic effect/No. treated
Leukopenia (wbc 3000 cells/mm³)	5/43
Thrombocytopenia (platelet count 100,000 cells/mm³)	6/43
Nausea, vomiting, diarrhea	8/43
Stomatitis	3/43
Paralytic ileus	2/43
Peripheral neuritis	4/43
Transient elevation in uric acid level	5/43
Alopecia	3/43
Anorexia	3/43

RESULTS AND COMMENT

Forty-eight patients were treated and antitumor effect was evaluable in 43 of these patients. Five patients were excluded because only the loading course of therapy was administered. Results were expressed as follows: complete response—a disappearance of all measurable lesions for 2 or more months; partial response—a decrease by 50% or more of measurable lesions for 2 or more months while no new lesions appeared; lesser response—a less than 50% objective response of measurable lesions.

Twenty of the 43 evaluable patients had prior radiation therapy and/or chemotherapy (radiation, radiation plus 5-FU, mechlorethamine, actinomycin D, 5-FU, adriamycin, and/or NSC-45388). Of these, a significant lesser response was observed in five of eight patients who received radiation or radiation and 5-FU, and one of seven patients who received 5-FU only.

The responses to combination chemotherapy are listed in table 1. Only four (9.3%) of the 43 evaluable patients had an objective response; none of these patients had received prior radiation therapy or chemotherapy. One of the four objective responses was a complete response of 6 months' duration. This was observed in a 16-year-old boy who had large metastatic bilateral pulmonary lesions after amputation of the left leg for osteogenic sarcoma. Subsequently, there was recurrence of the pulmonary lesions with rapid progression and death.

A lesser response was observed in ten of the 43 patients (table 1). These responses increased the performance status of these patients. A complete disappearance of a large pleural effusion and a 25% reduction in measurable lesions was observed in one patient with metastatic osteogenic sarcoma for 2 months, followed by progression of the disease and death.

The objective response rate of 9% in this clinical study of multiple-drug therapy is less than that noted in other reports of multiple chemotherapy for mixed groups of solid tumors. This may be because lesser amounts of agents were administered, although the toxicity encountered would appear to indicate that therapeutic levels were attained. It is our impression that the degree and number of responses in patients with carcinoma of the gastrointestinal tract are no greater than might be achieved with 5-FU alone. This combination or other combinations of chemotherapeutic agents may be beneficial in patients with metastatic osteogenic sarcoma when the tumor is, or becomes, resistant to adriamycin.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Adriamycin: NSC-123127

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)-methyl]methylamino]benzoyl]-

NSC-45388: 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide; CAS reg. No. 4342-03-4

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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6-Dehydro-6,17 α-dimethylprogesterone (NSC-123018) for the Treatment of Metastatic and Recurrent Ovarian Carcinoma [1,2]

George D. Malkasian, Jr., David G. Decker, Edward O. Jorgensen, and Maurice J. Webb [3]

SUMMARY

Therapy for ovarian cancer, stage II (International Federation of Gynecology and Obstetrics classification) or greater, at present includes total abdominal hysterectomy, bilateral salpingo-oophorectomy (when possible), radiation to the entire abdomen, and use of an alkylating agent. Synthetic progesterones have been given in small doses without significant responses, but treatment with a massive dose of progesterone appears to warrant further evaluation. In this report, the cases of two patients with objective tumor response in a group of nine patients treated with 6-dehydro-6,17α-dimethylprogesterone are briefly discussed.

[Cancer Chemother Rep 57:241–242, 1973]

Heretofore, when patients having metastatic and recurrent ovarian carcinoma failed to respond to surgical treatment, irradiation, and cytotoxic chemotherapy, no potential alternative therapy remained. However, nine such patients at the Mayo Clinic were treated with 6-dehydro-6,17α-dimethylprogesterone (NSC-123018) in 100-mg capsules taken orally, starting with 100 mg daily the first week, 200 mg daily the second week, 400 mg daily the third week, and 800 mg daily thereafter. This dose level is considerably higher than the level previously evaluated with other progesterones and with which less favorable results were obtained (1).

The cases of two of these patients are worthy of note since they represent objective responses for extended periods (30 and 45 months at latest followup) when, under previous circumstances, all palliative therapy would have been abandoned.

CASE REPORTS

Case No. 1.—A diabetic woman, 42 years of age at the time of this report, underwent dilatation and curettage, total abdominal hysterectomy, bilateral salpingo-oophorectomy, and appendectomy on November 2, 1968, for a bilateral papillary cystadenocarcinoma (endometroid type), grade 2, stage IIB (International Federation of Gynecology and Obstetrics [FIGO] classification). After she recovered from the surgical procedures, she received pelvic radiation (at another hospital) via 250-kilovolt equipment to a total tumor dose of 2500 rads. This treatment was followed by daily doses of 50 mg of cyclophosphamide [4] from February 3, 1969, to May 22, 1970, when obvious recurrence caused partial sigmoidal

[1] Received Mar 27, 1973; accepted Apr 26, 1973.

[2] NSC-123018: CAS reg. No. 977-79-7; pregna-4,6-diene-3,20-dione, 6,17-dimethyl-; medrogestone (USAN).

[3] Division of Obstetrics and Gynecology, Mayo Clinic and Mayo Foundation, Rochester, Minn.

[4] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

obstruction. On May 26, 1970, reexploration was done; this was followed by resection of a mass from the sigmoid colon. At that time, a few metastatic lesions in the pelvis were encircled with silver clips. On June 4, 1970, treatment with NSC-123018 was begun. On September 15, 1971, a mass was palpable in the pelvis, but on celiotomy no tumors were visible or palpable. The tissue encircled by the clips was excised. Tissue from one area had two microscopic foci of papillary adenocarcinoma, grade 2; the rest had only psammoma bodies with fibrous tissue. The mass palpated on pelvic examination proved to be only scar tissue with adherent intestine. The patient is alive, well, and apparently free of tumor 30 months after the beginning of treatment with NSC-123018.

Case No. 2.—Another patient, 48 years of age at the time of latest followup, underwent bilateral salpingo-oophorectomy for papillary cystadenocarcinoma, grade 2, stage III (FIGO), in March 1963. This procedure was followed by irradiation—a tumor dose of 2000 rads delivered to the total abdomen via cobalt 60 Tele-therapy. The patient was treated concomitantly with 400 mg of cyclophosphamide given intravenously daily for 4 days. One month after irradiation, daily treatment with orally administered 50-mg doses of cyclophosphamide was begun. On January 18, 1964, cholecystectomy was performed for cholelithiasis and an impacted stone in the cystic duct. At that time, several tiny nodules of calcified metastatic papillary adenocarcinoma were noted over the peritoneal surface. Since they were small, calcified, and few in number, daily treatment with 50-mg doses of cyclophosphamide was continued.

Reevaluation on June 6, 1968, indicated significant recurrence of disease in the pelvis. Laparotomy revealed significant spread to the mesentery, gutters, and large and small intestines. A further tumor dose of radiation (1500 rads) was delivered to the abdomen by a 6300-kilovolt linear accelerator; this was followed with daily oral treatment with 4 mg of melphalan.[5] This regimen was continued until January 4, 1969, when depressed leukocyte and thrombocyte counts as well as hemorrhagic cystitis necessitated its discontinuance. On March 19, 1969, a rapidly growing mass located posterior to the uterus filled half of the pelvis and several other small nodules were palpable in the pelvis. NSC-123018 was administered at the aforementioned dosage. The pelvic disease regressed to leave only a few small areas 1 cm in diameter. It remained so until March 2, 1971, when one lesion grew to 5 cm. Reexploration was followed by total abdominal hysterectomy, resection of the sigmoid colon and rectosigmoid with rectosigmoidostomy (end to end), and an ileodescending colostomy (end to side) with resection of the cecum and the terminal part of the ileum. Visible but not palpable tumor was left in the lower part of the pelvis. The tumor involved five areas in the resected specimen including the 5-cm pelvic mass and four others less than 1 cm in diameter. Part of the intestinal resection was necessitated by actinic changes. The patient has been receiving maintenance treatment with daily doses of 400 mg of NSC-123018 and is alive without palpable evidence of active tumor on January 6, 1973.

REFERENCE

1. WARD HW. Progestogen therapy for ovarian carcinoma. J Obstet Gynaecol Br Commonw 79:555–559, 1972.

[5] Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-.

ANNOUNCEMENTS

1973 Intra-Science Symposium: Nov 28–30, 1973

"New Ideas in Cancer Chemotherapy" is the subject of the Intra-Science Research Foundation's 7th annual major symposium, to be held November 28, 29, and 30, 1973, in Santa Monica, California. Sessions will cover Immunotherapy, Antimetabolites, Natural Products, Steroids, Radiation Sensitization, and Anti-Viral Agents. For information write to Conference Coordinator, Intra-Science Research Foundation, P. O. Box 430, Santa Monica, California 90406.

Medical Oncology Fellowships

Post-doctoral fellowships for practicing physicians are now available at the University of Southern California School of Medicine. The fellowships are for 1 month each and are tuition-free. They are designed for Board-eligible or certified internists; included are experience with standard and investigational agents, as well as special aspects of the care of patients on an oncology ward and clinic. Applicants should send a brief résumé to Dr. David Stolinsky, U.S.C. Oncology Service, 2825 South Hope Street, Los Angeles, California 90007.

Correction

Due to an oversight, two symbols are used interchangeably in the paper "In Vivo Determination of the Fractional Kill of Human Tumor Cells by Chemotherapeutic Agents" by Morris C. Berenbaum, which appeared in the October (Vol. 56, No. 5) issue of *Cancer Chemotherapy Reports*. Wherever the symbol Υ appears (in figures 1, 2, and 3), the symbol γ should be substituted. We apologize for this error.

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